Assessing the Impact of Oral Vitamin B12 Supplementation on Vibration Sensitivity, Dexterity, and Balance in Young Adult Vegetarians and Vegans

by

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ABSTRACT

Vitamin B12, found only in animal products, is a water-soluble vitamin important for DNA methylation, purine and pyrimidine synthesis, and the myelination of nerves. Symptoms of vitamin B12 deficiency include anemia, gait disturbances, altered vibration proprioception, impaired vision, psychosis, depression, dementia-like illness, and ultimately death. Because vegetarians and vegans consume fewer animal products in their diet than omnivores, they are inherently more at risk for developing these symptoms of vitamin B12 deficiency. Thus, the purpose of this study is to examine the correlation between nervous system markers (balance, dexterity, and vibration sensitivity) and markers of vitamin B12 nutriture (serum B12 and serum holo-transcobalamin II) in a cross-sectional study (n=38). In addition, the impact of daily oral vitamin B12 supplementation on these markers in an 8-week randomized controlled trial was also examined (n=18). The results of the cross-sectional study revealed a moderate correlation (R=-0.351, p=0.031) between serum B12 and left-hand functional dexterity. The results of the intervention study revealed no significant time*group interactions for markers of nervous system functions and biochemical values (after the removal of outliers). In addition, the time*group interaction appeared to be larger for those individuals with a baseline serum B12 of less than 303 pmol/L. These results suggest that vitamin B12 supplementation may have a more pronounced effect on those individuals who are in a state of vitamin B12 depletion (<303 pmol/L serum concentration). In addition, the results also suggest that 8 weeks of oral supplementation is not a long enough period to create significant clinical change, and it is likely that improvements in neurological measures would require long-term supplementation.

DEDICATION

This dissertation is dedicated to my husband and family for their unwavering support.

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CHAPTER 1

INTRODUCTION

Overview

Current research examining the prevalence of vitamin B12 (cobalamin) deficiency among vegetarians is extremely varied. In a 2013 meta-analysis examining deficiency prevalence in 18 different studies, deficiency rates in vegetarians and vegans ranged from 0-86.5% [1]. In studies where the mean or median age is between 22 and 29 years, the deficiency rate of lacto-ovo vegetarians ranges from 6% to 31% [2-4]. These rates of deficiency in the vegetarian population are higher than the national rate of 1.7% for 19-30 year-olds [5] due to the lack of B12 in plant-based food [6]. Other populations at risk for B12 deficiency include the elderly, bariatric surgery patients, those with a malabsorptive disease such as inflammatory bowel disease [7], and patients chronically taking antacids, Metformin (diabetes) [7], omeprazole (gastro esophageal reflux disease), and neomycin (antibiotic) [8]. Further, those with generally poor nutrition [9], including alcoholics and eating disorder patients, are at risk for vitamin B12 deficiency.

This prevalence of deficiency is problematic because vitamin B12 is necessary for vital nervous system functions, including the synthesis of neurotransmitters and myelin sheaths. This myelination may be important for hand dexterity as the literature suggests possible connections between dexterity and myelination in adolescent-onset psychosis patients [10]. Dexterity is also linked to right cerebral white matter (e.g. bundles of myelinated axons in the brain [11]) and primary motor cortex volume in healthy older adults [12]. Reduced hand functionality can decrease quality of life by negatively impacting daily activities, personal medical care, or various activities related to work

performance [13]. Subtle changes in hand dexterity may reveal subclinical deficiency which, if identified earlier, can aid in the prevention of serious complications of vitamin B12 deficiency such as impaired vision, personality changes [7], psychosis, depression, mania [8], and dementia-like illness [9].

Despite a large amount of research regarding the impact of vitamin B12 deficiency on markers of cognitive health, there is a *gap in the knowledge base* regarding the effect of vitamin B12 status on functional markers (such as dexterity, vibration sensitivity, and balance) of neuropathy in any population, including vegetarians.

In micronutrient deficiency research, there is a common theme of examining the consequences of severe deficiency and largely ignoring vitamin depletion states, i.e. the low-end of the 'normal' spectrum. Just as with many other vitamins, this matter persists in the realm of vitamin B12 research. Irreversible neurological symptoms often appear in vitamin B12 deficiency [14], however, there is little research that has examined the very early and subtle signs of the onset of these symptoms. Young adult vegetarians are an excellent model to examine the subtle onset of neurological consequences of B12 deficiency, as they are more likely to have low or deficient vitamin B12 status than the average population. Furthermore, the neurological consequences normally associated with aging will likely not be present in this young population (unless other underlying medical conditions are at play). This research will examine, using an 'at-risk' population, whether subtle changes in dexterity are measurable and relate these changes to vitamin B12 status.

In addition to understanding whether vitamin B12 status is associated with subtle changes in neurological function, it is not known whether vitamin B12 supplementation

improves neurological function in a population at risk for vitamin B12 deficiency, such as vegetarians. An intervention study such as this will allow us to identify if subtle neurological consequences of vitamin B12 respond to supplementation in a vegetarian population. According to the 1998 Dietary Reference Report, the onset of neurological symptoms in vitamin B12 deficiency is gradual [15]. However, according to this report, the RDA is based on the amount of B12 needed to prevent anemia and maintain "normal plasma levels" [15], rather than the prevention of subtle neurological symptoms (as research in this area was lacking when the report was created). Thus the trend line used to create the RDA that describes the relationship between vitamin B12 consumption and the presence of anemia is based solely on hematological symptoms and does not take into account the neurological symptoms that often accompany vitamin B12 deficiency. Further, according to Briani et al., neurological symptoms often manifest prior to hematological symptoms [8]. If subtle neurological symptoms present in individuals with depleted or low (yet normal) nutriture states, then the quality of life can be negatively affected. It is important to assess if markers of neurological decline positively respond to vitamin B12 supplementation, as this is an inexpensive and easy to access therapy. In addition to improving quality of life, the timely identification and remedy of subtle neurological symptoms may reduce the financial consequences of vitamin B12 deficiency, as consequences of neuropathy have the potential to contribute to decreased work productivity. Furthermore, individuals with permanent neurological damage can accrue extensive medical expenses throughout their lifetime. It is essential to explore and identify functional declines in populations at risk for B12 deficiency, as well as the

neurological consequences of and solutions to vitamin B12 deficiency, as cognitive and functional declines may have a large impact on quality of life.

Purpose of Study

The *goal* of this research is to understand the impact of deficient, depleted, and normal vitamin B12 status on previously unstudied neurological markers in healthy adult vegetarians and vegans. In addition, we will also investigate a cause and effect relationship between vitamin B12 supplementation and improvements in dexterity, vibration sensitivity, and balance. Collectively, the outcomes of this study will advance the field of nutrition and micronutrient metabolism by defining previously unstudied neurological consequences of vitamin B12 deficiency. This study will *lay the foundation* for understanding the physiological consequences of subclinical vitamin B12 deficiency. The *long-term goal* of this research is to encourage the reexamination of the vitamin B12 RDA guidelines.

Research Aim and Hypothesis

Our *central hypothesis* is that a vitamin B12 supplement intervention will be successful in improving vibration sensitivity, balance, and dexterity in young adult vegetarians. This hypothesis has been determined based on evidence in the literature suggesting a possible link between vitamin B12, decreased myelination, and poor dexterity. The *rationale* for our hypothesis is to discover previously unknown consequences of vitamin B12 deficiency in young adults, as well as to discover if these consequences are also subtly present in those with depleted or normal B12 nutriture. The *specific aims*, which support the overall objective of the project, are as follows:

1. **To examine correlations between vitamin B12 and dexterity (Purdue Pegboard Test, Functional Dexterity Test), vibration sensitivity (Physitemp Vibratron II), and balance (AMTI Accusway) in healthy, young vegetarians and vegans.** Due to the importance of vitamin B12 in nervous system function, we hypothesize that dexterity, vibration sensitivity, and balance will have a direct correlation with serum cobalamin concentrations.
2. **To identify changes in dexterity (Purdue Pegboard Test, Functional Dexterity Test), vibration sensitivity (Physitemp Vibratron II), and balance (AMTI Accusway) in a vitamin B12 supplement (500 mcg 1x day) intervention group compared to a placebo control group (vinegar pill 1x day)**. Due to the impact of B12 nutriture on myelination, and the association between poor myelination and neuropathy, we hypothesize that dexterity, vibration sensitivity, and balance scores will improve in the intervention group, and no change will be detected in the control group.

Definition of Terms

Vegan: An individual who consumes no meat or fish, and consumes dairy or egg-containing products less than once a week.

Lacto-ovo Vegetarian: An individual who consumes meat (i.e. fish, poultry, pork, beef, or any other animal product excluding dairy or eggs) less than once a week, but consumes eggs or dairy products (i.e. milk, yogurt, cheese) at least once a week

Omnivore: An individual who consumes meat or fish at least once a week.

Dexterity: Fine motor skills of the hands

Vitamin B12: In this text, 'vitamin B12' and 'B12' will be used interchangeably. Vitamin B12 is also known as cobalamin (Cbl).

Limitations

There are 5 major limitations for this study: (1) the restricted population, (2) the lack of administering multiple types of vitamin B12 supplements, (3) the lack of a dose-response component, (4) the limited time period in which the study will be conducted, (5) the lack of a crossover design, and (6) a small sample size.

1. Vegetarians were chosen as the target population because they are more likely to be deficient than an individual from the general population. However, this limits the external validity, as there may be other unknown lifestyle factors associated with vegetarianism that could affect the outcome variable.
2. Although we will be testing the efficacy of an oral pill form vitamin B12 supplement on changes in dexterity and nerve conduction speed, we are not examining the effects of sublingual supplements, nasal sprays, or intramuscular injections. Thus, the generalizability of the results of the study would not span to all forms of vitamin B12 supplements.
3. This study will only contain one intervention group, rather than multiple groups to test for a dose-response. This also limits the generalizability of the results, as any changes (or lack of changes) in the outcome variable would only be attributed to the dosage used in this particular study.
4. This study will be conducted over a period of 8 weeks with a single pre- and post-test. It is possible that a continued vitamin B12 supplement intervention would have additional effects after the specified 8-week period.

5. This study design is parallel in nature, rather than a crossover. This is due to the storage of vitamin B12 in the liver for extensive periods of time. Thus, a crossover design is often unfeasible for vitamin B12 supplement intervention studies. If this study was conducted as a crossover study, the washout period would need to be at least 5 years in length.
6. Although the ideal sample size for this study is 54 subjects, only 18 subjects completed the intervention portion of the study. Thus, this study was underpowered.

CHAPTER 2

REVIEW OF LITERATURE

Introduction to Vitamin B12

Vitamin B12 is a water soluble vitamin [16] that is synthesized by bacteria found in the intestinal tract of animals [17]. B12 is important for some metabolic and neurologic functions in the body including cell division, DNA synthesis, folate metabolism, homocysteine metabolism, red blood formation, and myelination of nerve cells [14]. Sources of B12 include animal products, such as milk, meat, eggs and fish [18]. B12 can also be found in fortified foods or plant products contaminated with B12 producing bacteria [17]. Vitamin B12 absorption into the bloodstream occurs due to a combination of high stomach acidity and the availability of intrinsic factor [14]. After absorption, metabolically active vitamin B12 is transported in the blood by transcobalamin II (TCII). Vitamin B12 can also be free in the blood, however, this form of B12 is not metabolically active.

Because animal products are the major source of vitamin B12 in an individual's diet, vegans and vegetarians are particularly at risk for developing a B12 deficiency. In addition, the elderly are at a high risk for B12 deficiency due to a high incidence of achlorhydria, a condition that weakens stomach acid [14]. Symptoms of vitamin B12 deficiency include changes to blood and bone marrow cells, as well as neurological and cognitive disorders [14]. Although symptoms of vitamin B12 deficiency are often severe, deficiency will often take several years to become symptomatic due to large stores of vitamin B12 in the body [14]. According to the National Institutes of Health (NIH), adults should consume at least 2.4 ug of vitamin B12 daily [19] (table 1). However, the typical

daily consumption often exceeds this amount as the median daily consumption for females is 3.5 μg, and the median daily consumption for males is 5 μg [7].

Table 1: Vitamin B12 Recommended Dietary Allowances (RDAs) by Age Group. All values obtained from NIH [19].

Population	Vitamin B12 RDA (μg)
0-6 months	0.4
7-12 months	0.5
1-3 years	0.9
4-8 years	1.2
9-13 years	1.8
14-18 years	2.4
Adults	2.4
Pregnant women	2.6
Breastfeeding women	2.8

Absorption, Transport, Uptake, and Storage of Vitamin B12

The absorption and transport of vitamin B12 rely heavily on several proteins including intrinsic factor (IF), trans-cobalamin II (TCII), and hepatocorrin (HC). The absorption process begins in the stomach, where gastric pepsin causes B12 to be released from proteins in food. Next, vitamin B12 binds to hepatocorrins, which are proteins released in the saliva [20]. In addition, gastric acid in the stomach also stimulates the parietal cells of the stomach to release intrinsic factor, which has a strong affinity towards B12 and does not bind well with its analogues [21]. Intrinsic factor binds with vitamin B12 in the small intestine, where the bond between hepatocorrins and B12 is hydrolyzed by pancreatic proteases [20]. The IF and vitamin B12 complex then binds to receptors on enterocytes in the distal ileum. Next, the complex is absorbed and B12 is released into circulation bound to TCII. In addition, about 1-3% of vitamin B12 can be absorbed into the enterocytes via passive diffusion [22].

Although the intercellular path of vitamin B12 within the enterocytes is still poorly understood [21], it is believed that there is a cellular, non-lysosomal vesicle that houses endogenously produced TCII, and provides the location for TCII-B12 complex formation. The method for vitamin B12 entry into this vesicle, however, is unknown [23]. In addition to this receptor-mediated enterocyte absorption, high doses of B12 can cause minimal absorption into circulation by diffusion [20]. After the IF-B12 compound is taken up by enterocyte cells via endocytosis, the complex is broken down within the lysosome. Intrinsic factor is then degraded. Vitamin B12 is transferred to a separate vesicle, where it binds with TCII, and then is released into circulation via transcytosis [23]. It is essential for TCII to be continuously produced in the body, as its half-life is only about one hour [24].

TCII is essential to vitamin B12 metabolism and carries 10-30% of vitamin B12 in the body [21]. A small number of studies have found that the presence of the receptor for the TCII-B12 compound, TCb1R, is associated with the cell cycle. This is believed to be due to the high need of vitamin B12 during DNA synthesis [21].

Circulating TCII-Cbl is distributed among body cells via circulation and is absorbed into body cells via receptor-mediated endocytosis [23]. Once in the cell, the TCII-Cbl complex is taken up by a lysosome, where the TCII protein is degraded. Free vitamin B12 is then released, where it will function in its coenzyme form [23].

Vitamin B12 is also found free in the blood, as well as bound to hepatocorrins. Hepatocorrins, which released in the saliva, are also known in the literature as R-binder and TCI or TCIII [21]. Although no concrete function for these compounds has been defined by the literature, the fact that HCs do not have an affinity as precise as TCII and

IF for vitamin B12 suggests that hepatocorrins may prevent the assimilation of B12-like compounds into the vitamin B12 metabolic pathway. In addition, these compounds may also serve to bind to free B12 and prevent the loss of free B12 from circulation [21]. In addition to this, vitamin B12 can also be reabsorbed during the excretory process (known as the enterohepatic pathway). Significant amounts of vitamin B12 are released with bile, however a large amount binds with intrinsic factor in the small intestine to minimize loss in feces and urine. Vitamin B12 released from sloughed intestinal cells is also reabsorbed in the same manner [7].

Vitamin B12 is stored primarily in the liver, where up to 3 mg can be stored. This amount is sufficient for up to 5 years of use at adequate plasma concentrations [20]. Thus, only individuals who fail to consume vitamin B12 for an extended period are likely to develop symptoms of deficiency.

Transcobalamin II

Evidence of protein bound to circulating B12 was first discovered in the late 1950s and early 1960s. However, it wasn't until 1972 that this substance, transcobalamin II, was first purified for the purpose of studying the chemical properties in detail [25]. Although almost 55 years have passed, the metabolic pathways of transcobalamin II, the carrier of active vitamin B12, are still not fully documented in the literature [25,26]. Further, although some details are known about the particular genes that code for TCII, not much is known regarding the specific metabolic processes that regulate the synthesis of this essential molecule.

The TCII gene (TCN2 [26]) is located on chromosome 22 [25]. This gene does not contain a TATA promoter region, unlike the genes for intrinsic factor and hepatocorrin.

Genes without this factor are thought to produce protein at a relatively regular rate, as the TATA box is a genetic regulatory element [25]. However, some studies have demonstrated an increase of serum TCII in response to both oral [25] and intravenous [27] vitamin B12 supplementation. Some researchers believe this response to increased serum B12 is due to a 5' flanking DNA sequence that acts as a regulatory element [25].

Although many studies have explored the TCII synthesis ability of various tissues, there is yet another lack of consensus in the literature as to the predominant site of production. Animal cell studies have yielded evidence of TCII production in the liver, peritoneal macrophages, and ileum. However, an additional study examining hepatectomized dogs did not document a difference in TCII production, suggesting that the liver is not the main site for transcobalamin-II synthesis [25]. The human cell studies have yielded evidence of TCII production in liver tumors, fibroblasts, bone marrow cells, and umbilical cord vein endothelial cells [25]. Rothenberg and Quadros suggest venous endothelial cells as the predominant site for TCII synthesis. Because vitamin B12 is delivered to tissues (where it is ultimately destroyed [23]) via the circulatory system, arterial circulation will naturally have a higher concentration of TCII than venous circulation. Thus, Rothenberg and Quadros proposed that this insufficient concentration of TCII stimulates production from venous endothelial cells. Due to the large surface area of the venous endothelium, these cells are more likely to efficiently replenish TCII [25]. However, this study was conducted using human umbilical cord vein cells, which may behave differently from other venous endothelial cells.

Although research has largely ignored the synthesis and regulation of TCII, much recent research has been devoted to TCII receptor regulation. TCII receptors are present

in various tissues such as the heart, lungs, small intestine, spleen, liver, and kidneys [25]. As vitamin B12 is an important substance for DNA synthesis and DNA methylation, B12 uptake occurs in all types of cells [23]. The TCII-B12 complex binds to the TCII receptor in a biphasic reaction that is temperature and pH dependent, and requires divalent cations such as Ca^{2+} or Mg^{2+} [25]. The TCII-Cbl complex moves into the cell via receptor-mediated endocytosis, after which the TCII receptor can be recycled [25].

Metabolism of Vitamin B12

The four biologically significant cobalamins are hydroxycobalamin, adenosylcobalamin, methylcobalamin, and cyanocobalamin. Most vitamin B12 in food comes in the form of hydroxycobalamin, whereas most B12 in supplements comes in the form of cyanocobalamin. The remaining two vitamin B12 vitamers are adenosylcobalamin and methylcobalamin, which are the metabolically active forms of vitamin B12 [7]. The metabolism of vitamin B12 can be divided into two separate categories based on the location of activity: cytoplasm or mitochondria. Once vitamin B12 is absorbed into the cell, it is converted to either adenosylcobalamin or methylcobalamin, which act as coenzymes for two essential metabolic processes in the body [21].

Vitamin B12 metabolism in the cytoplasm utilizes methylcobalamin, one of the active vitamin B12 vitamers, as the cofactor for the methionine synthase enzyme. This process, also known as the methylation cycle, serves numerous metabolic functions [20]. The methionine synthase enzyme serves to convert homocysteine to methionine. Methionine is then converted (using ATP) to S-adenosyl methionine, which acts as a methyl donor for DNA methylation. This process of DNA methylation is important in

the epigenetic modification of gene expression [20]. During this methyl donation, S-adenosyl methionine becomes S-adenosyl homocysteine, which is then converted back to homocysteine (Hcy).

This methionine synthase enzyme action also plays a major role in the folate cycle. The conversion of homocysteine to methionine requires a methyl donor, 5-CH3-tetrahydrofolate. During this methyl donation, 5-CH3-tetrahydrofolate becomes tetrahydrofolate (THF) [20]. Thus, folate is the methyl donor for this portion of the cycle. The production of THF is necessary for purine synthesis and the next step in the folate cycle (methyleneTHF) is essential for pyrimidine synthesis [20]. These purines and pyrimidine nucleotides are the main components of DNA, RNA, and nucleoside triphosphates (such as ATP) [20]. Thus, not only is methylcobalamin essential for proper DNA and RNA synthesis, but it is also essential for controlling the expression of this genetic material.

Vitamin B12 metabolism in the mitochondria utilizes adenosylcobalamin, the second active vitamin B12 vitamer, as the cofactor for methyl malonyl-CoA mutase enzyme. This vitamin B12 dependent enzyme is essential for the breakdown of propionyl-CoA, a three carbon compound which results from the breakdown of odd-numbered chain fatty acids during β-oxidation in the mitochondria [20]. This propionyl-CoA is converted to methyl malonyl-CoA, which is then converted to succinyl-CoA by methylmalonyl-CoA mutase [20]. The resulting succinyl-CoA can then enter into the TCA cycle for energy production. A lack of B12 thus results in the inhibition of the methyl malonyl-CoA mutase enzyme and buildup of methyl malonyl-CoA (MMA CoA) and methylmalonic acid (MMA). Further, because the rate of this enzymatic process is

regulated by the concentration of products produced after the succinyl-CoA metabolic step, a buildup of MMA CoA inhibits the transfer of long-chain fatty acids into the mitochondria. Thus, this leads to an abnormal accumulation of these fatty acids in the cytoplasm [20].

Applications in Heart Disease

Vegetarian diets have been found to improve several biochemical markers including as C-reactive protein, oxidative stress, blood glucose, and lipid profiles; increases in all of which are associated with an increased risk for heart disease [28]. In addition, vegetarian diets are also protective against arterial plaque formation, associated with lower blood pressure (both systolic and diastolic), and associated with a lower prevalence of overweight and obese individuals [28]. Contrary to what is expected, however, these lowered risk factors do not always translate to a decreased prevalence of heart disease in vegetarians and vegans.

Surprisingly, some vegetarian and vegan populations examined in the literature displayed an increased prevalence of heart disease when compared to omnivores [28]. For example, a 2013 paper reporting and reviewing preliminary results from the EPIC-Oxford study (n=55,041) found a standardized mortality ratio (SMR) for circulatory disease to be 38% in non-vegetarians, but 40% in vegetarians [29]. In addition, the SMR for cerebrovascular disease was found to be 41% in non-vegetarians and 52% in vegetarians. However, this study did find a higher all cause mortality in non-vegetarians (SMR = 39%) than in vegetarians (SMR = 40%) [29]. The participants in this study were predominantly female (78% of the non-vegetarians and 75% of the vegetarians). The median age for the non-vegetarians in this study was 47 years old, while the median age

for the vegetarians was 36 years old at baseline [29]. The Adventist Health Study 2, which examined mortality in vegetarians and vegans in 73,308 participants, also found an increased risk for death due to vascular disease in certain vegetarian or vegan cohorts [30]. The hazard ratio of death due to ischemic heart disease was 1.39 in female vegans, and 1.09 in female semi-vegetarians compared to the reference population of female non-vegetarians (hazard ratio = 1). The hazard ratio of death due to cardiovascular disease was 1.18 in female vegans compared to non-vegetarians [30]. None of the hazard ratios for vascular disease were higher than that of the reference population in male vegetarians or vegans. However, in the overall populations, the hazard ratio of death due to vascular conditions is lower than that of the non-vegetarian reference population [30]. In this study, the mean age of the dietary groups ranged from 55.9 years to 58.8 years old. In addition, the prevalence of females ranged from 63.8% to 69.7% of the sample size [30]. Although the risk of some types of cardiovascular disease may be higher in certain subsets of the vegetarian population, the overall risk of circulatory health problems in the vegetarian and vegan populations tend to be lower than the omnivorous population [28-30].

Pawlak suggests, however, that vegetarian diets are not as protective against vascular disease as previously thought [28]. The reason for this inconsistency between theory and observation is likely due to high serum homocysteine concentrations commonly present in vegetarians and vegans. A recent meta-analysis of 21 studies examining hyperhomocysteinemia in in vegetarian adults found an average homocysteine concentration above 15 μmol/L (the most conservative definition of hyperhomocysteinemia) in vegetarians or vegans in 11 of the studies [28]. There is ample evidence in the literature that documents an association between increased serum

concentrations of homocysteine and an increased risk of heart disease [4,28,31-33], particularly venous thrombosis, coronary heart disease, stroke [32], and extracranial carotid artery stenosis [28].

Homocysteine is thought to contribute to this increased risk of heart disease primarily by atherogenesis. Acute hyperhomocysteinemia is associated with decreased nitrous oxide synthesis, increased LDL oxidation, and endothelial inflammation, and increased foam cell formation; while chronic hyperhomocysteinemia is associated with decreased superoxide dismutase activity, aortic calcification [28]. All of these metabolic changes contribute to the development of cardiovascular disease. Vitamin B12 deficiency, independent of hyperhomocystenemia, can contribute to circulatory problems via B12 induced macrocytosis (abnormally large blood cells), and have been shown to increase the risk for fatal coronary disease, fatal and nonfatal myocardial infarction, stroke, and symptomatic heart failure [28]. Thus, vitamin B12 deficiency contributes to an increased risk for heart disease using several metabolic processes.

Homocysteine, or tHCY, is a metabolite in the methylation cycle of vitamin B12 metabolism. As mentioned above, tHCY is converted to methionine by methionine synthase, a vitamin B12 dependent enzyme [20]. When vitamin B12 serum concentrations are low, as is common in vegetarians, methionine synthase does not function at full capacity. Consequentially, homocysteine concentrations tend to be higher in populations at an increased risk for vitamin B12 deficiency, such as vegetarians and vegans. Using the data from the 3rd NHANES study, hyperhomocysteinemia (high homocysteine concentration) is defined as ≥11.4 μmol/L for men and ≥10.4 μmol/L for women [28]. Suboptimal methionine synthase enzyme activity can also be due to inadequate serum

folate, as folate acts as a methyl group donor in the methionine synthase reaction. Thus, folate deficiencies can also cause increased serum homocysteine concentrations. However, hyperhomocysteinemia is more likely to be caused by low vitamin B12 than low folate. In one study, 0.3% of the risk for high tHCY was credited to low folate, whereas 29.7% was attributed to low serum B12 and 36.4% to inadequate TCII [28].

Among the general population, hyperhomocysteinemia typically ranges in prevalence from 9-15%, with higher rates in female adolescents and elderly women [28]. However, similar to the research examining the rates of vitamin B12 deficiency in vegetarian and vegan populations, a 2015 meta-analysis found prevalence rates of hyperhomocysteinemia to be between 12 and 78% of the vegetarian and vegan populations [28]. Results from Pawlak's meta-analysis showed, however, that 13 of the 20 studies examined documented a hyperhomocysteinemia prevalence above 50% in vegetarian and/or vegan populations [28].

According to the Hordaland Homocysteine Study, increased serum homocysteine concentrations are associated with a variety of other demographic characteristics in addition to the diet. These include the male gender, age, physical inactivity, the number of cigarettes smoked per day, coffee intake (cups/day), high blood pressure, and high cholesterol (although these last two associations were weak) [32]. Refsum et al. also found that three of these factors combined had a much more significant effect on tHCY concentration than did one factor alone. However, investigators also noted that lifestyle changes, such as smoking cessation and increased folate intake, can reduce tHCY concentrations [32]. Thus, although high homocysteine concentrations are a consequence

of inadequate vitamin B12 consumption, these elevated values can also be due to other unrelated factors.

Few studies have examined the impact of utilizing B vitamin supplementation to decrease serum homocysteine concentrations, with the ultimate goal of reducing the risk of heart disease. However, many studies have been conducted to examine the effectiveness of vitamin B12 supplementation to simply reduce tHCY levels [32]. More specifically, B vitamin supplementation is a commonly used treatment for homocystinuria (excessive serum tHCY) due to cystathionine β-synthase deficiency, which will eventually lead to death if untreated [32]. Thus, although high tHCY levels have the potential to diminish protective effects of a vegetarian diet against heart disease, it is possible that supplementing the diet with vitamin B12 and folate (if inadequate) may mitigate any negative effects that homocysteine concentrations have on vascular disease risk in vegetarians and vegans.

Folate and Vitamin B12 Interaction

Because the major cause of megaloblastic anemia is a decrease in methyleneTHF, folate deficiency, in addition to vitamin B12 deficiency, can also manifest in megaloblastic anemia. Thus, unless serum concentrations of folate and vitamin B12 are examined, it is impossible to determine which vitamin deficiency is the cause of megaloblastic anemia [8]. This is problematic because, as early case reports demonstrate, B12 deficiency was often mistaken for folate deficiency and treated only with folate. As a result, there is alleviation of the anemia-like symptoms, but no alleviation of neurological symptoms [34]. Thus, folate supplementation is known to affect the symptoms related to a vitamin B12 deficiency. However, there is a large gap in the literature

documenting the consequences of folate supplementation in B12 deficient individuals (in both human and animal subjects) [34]. Thus, how folate impacts the presentation of vitamin B12 deficiency symptoms is still debated in the literature. It is accepted that supplemental folate (folic acid) masks macrocytic anemia symptoms, but it is largely debated whether high supplemental folate exacerbates neurological symptoms in vitamin B12 deficiency [35,36]. In this masking effect, high consumption of folic acid can change the distribution of cellular folate. This leads to the un-methylated folic acid bypassing the need for the conversion of methyltetrahydrofolate, and continue the process for DNA synthesis [34].

Although the exact mechanism for heightened neurological degradation in vitamin B12 deficient but folate surplus individuals has only begun to be revealed in the literature; there is an increasing amount of evidence that high folate levels in individuals with low vitamin B12 status are associated with cognitive impairment [34]. This association, however, is not present in individuals with normal vitamin B12 nutriture. In fact, in individuals with normal vitamin B12 nutriture, a high plasma folate concentration was found to have a protective effect on cognition [34]. Selhub et al. suggest the reason for an increased neurological degradation in the presence of high folate is worsening enzymatic function of B12 in vitamin B12 deficient individuals [34]. This study examined NHANES data on folate, vitamin B12, MMA, and total homocysteine (tHcy) in otherwise healthy adults 20 years and older. The presence of high plasma folate in this study was attributed to vitamin supplements and folate fortification of food[34]. Results revealed that, in individuals with low vitamin B12, there was a 'U' shaped curve for tHcy and MMA data. Data showed an intermediary range where tHcy and MMA values were

normal, but values increased in the presence of both low and high folate [34]. These results were not entirely expected as folate only directly interacts with the portion of vitamin B12 metabolism (methylcobalamin) that results in elevated tHcy levels. Thus, although Selhub et al. states that high folate concentrations must also affect the activity of adenosylcobalamin, they also acknowledge that it is difficult to imagine how this occurs.

Selhub et al. suggests two possibilities for the theory that high folate in vitamin B12 deficient individuals exacerbates neurological consequences. One hypothesis of how folate may interact with the methionine synthase (MS) and methyl-malonyl CoA mutase (mutase) enzymes, which Selhub acknowledges is unlikely, is an interaction with the reduction of cob(II)alamin to cob(I)alamin. This process occurs in both metabolic processes of vitamin B12 metabolism, but is drastically different in each part of the cell [34]. A second hypothesis Selhub suggests that folate may interact with both the MS and mutase enzymes is in the same way that nitrous oxide inhibits both processes. Although it is not known exactly how nitrous oxide affects the mutase enzyme, people exposed to high amounts of inhaled nitrous oxide have increased tHcy and MMA [34]. As evidenced by the decreased activity of adenosylcobalamin when exposed to nitrous oxide, the activity of adenosylcobalamin and methylcobalamin may impact each other in some way [34]. This second hypothesis, according to Selhub et al., is more likely. Thus, the exacerbation of clinical symptoms of vitamin B12 deficiency in the presence of high plasma folate is thought to be due to an interruption of normal metabolic vitamin B12 functioning in the mitochondria, similar to nitrous oxide toxicity, resulting in higher concentrations of tHcy and MMA [34]. Due to this interaction between folate and vitamin

B12, it is essential to measure and possibly control for plasma folate concentrations when examining vitamin B12 in research studies.

Biochemical Assessment Methods

Interest in the medical benefits of vitamin B12 began in 1926, when George Minot and William Murphy discovered that pernicious anemia, a complication of B12 deficiency, could be treated by feeding inflicted patients a diet high in liver [14]. Since then, a number of assessment tools have been developed to measure an individual's vitamin B12 status and assess risk for deficiency. Three available measurement tools to assess vitamin B12 status are total serum B12, serum TCII, and urinary methylmalonic acid (MMA). Serum TCII is the earliest indicator of B12 stage 1 negative balance (<40 pg/mL), where a normal TCII concentration is >50 pg/mL [6]. Serum TCII is an excellent indicator of B12 status because it only transports metabolically active forms of B12. Total serum B12 is a late stage indicator of B12 deficiency, where normal serum B12 is 300-300 pg/mL [6]. This is because an individual can be deficient in metabolically active B12, yet still have significant quantities of free B12 in the blood. Urinary MMA, which is inversely associated with B12 intake, is considered the best marker of the extent of low B12 status, once deficiency has been reached. Vitamin B12 deficiency is indicated by a urinary MMA concentration >271 nmol/L [1].

Because each of the available B12 assessment tests appear to function best in individuals with different B12 concentrations, there is no consensus in the field of medicine as to which test should be used as the universal biomarker for B12 deficiency [14]. This dilemma is particularly complicated by the wide range of B12 concentrations that different diet categories (vegan, vegetarian, and omnivore) can produce. In addition,

although TCII appears to be the earliest indicator of vitamin B12 deficiency; there is no clear consensus in the literature as to the clinical implications of low TCII [24]. Further, as TCII needs to be continuously produced due to its short half-life, this functional marker may not be the best indicator of long term B12 status. The problem with short half-life in TCII is similar to that of 1,25-dihydroxyvitamin D, which has a half life of 4-15 hours [37]. According to the Institute of Medicine, this active form of vitamin D is not the ideal method to measure vitamin D status due to the strict regulation of this compound [38]. It is possible that a similar mechanism may be occurring with TCII. More research is needed regarding the regulation of TCII and the impact of its short half-life on its value as an indicator of vitamin B12 nutriture.

Vitamin B12 Deficiency: Populations at Risk

Current research examining the rate of vitamin B12 (cobalamin) deficiency among vegetarians is extremely varied, citing deficiency rates ranging from 0-86.5% [1]. In studies where the mean or median age is between 22 and 29 years, the deficiency rate of lacto-ovo vegetarians ranges from 6% to 31% [2-4]. These rates of deficiency in the vegetarian population are higher than the national rate of 1.7% for 19-30 year-olds [5] due to the lack of B12 in plant-based food [6].

Table 2. Amount (µg) of vitamin B12 in common food sources. Nutrient information obtained from the USDA SuperTracker™ website [39].

Food Source	µg of B12	Percent of Adult RDA
Beef liver (braised, 3oz)	58.8	2450%
Clams (1 medium, raw)	7.2	299%
Hamburger patty (¼ lb., less than 80% lean)	2.2	92%
Cheerios® cereal (1 cup)	1.9	78%
Milk, reduced fat (2%, 1 cup)	1.3	54%
Cheddar cheese (¼ cup shredded)	0.2	10%
Tofu (1 cup, ½" cubes)	0.0	0%

Other populations at risk for B12 deficiency include the elderly, bariactric surgery patients, those with a malabsorptive disease such as inflammatory bowel disease [7], and patients taking chronically taking antacids, Metformin (diabetes) [7], omeprazole (gastro esophageal reflux disease), and neomycin (antibiotic) [8]. Further, as those with generally poor nutrition are at risk [9], alcoholics and eating disorder patients may also develop vitamin B12 deficiency. Thus, as vegetarians and older adults are the two largest population groups without inherent medical conditions which are likely to be deficient in vitamin B12, they are ideal groups to conduct research regarding the consequences of vitamin B12 deficiency.

Vitamin B12 Deficiency in Vegetarians and Vegans

Vegetarians and vegans are more likely to become deficiency in vitamin B12 than an omnivore population because vitamin B12 is only found in animal sources, which are decreased or absent in vegetarian and vegan diets. Although there are several studies examining the vitamin B12 status of vegetarians [40-43], there are no large studies examining the prevalence of vitamin B12 deficiency in young adult vegetarians and vegans. Out of eighteen studies included in a 2013 review examining vitamin B12 deficiency, none focused on a young adult population [40]. The results of this review cited deficiency prevalence ranging from 11% to 86% [40]. The age, diet duration and assessment criteria used in this study varied widely, depicting the need for a large population based study with clearly defined population parameters and assessment criteria. Most research in this area is focused on deficiency rates in older adults, children, and pregnant women. According to a recent review by Pawlak et al., the individuals

within this diet category who are most at risk for deficiency were those who did not consume supplements or fortified foods [1]. In addition, those who were considered 'strict' vegans were more likely to be deficient than 'moderate' vegans [1]. Thus, due to the increased risk for deficiency, along with a lack of decreases in dexterity associated with aging, young adult vegetarians are an ideal population to examine the effects of vitamin B12 deficiency on dexterity.

In a 2009 review published in the American Journal of Clinical Nutrition, Elmadfa and Singer examined vitamin B12 nutriture on a global level by analyzing 16 studies. Results from this review identified two studies from Germany (n=186, n=131) and one study from India (n=204) where the mean serum concentration of vitamin B12 was less than 148 pmol/L in vegetarians or vegans [42]. In all of the 16 studies cited in this review, the stricter diet types (i.e. vegans, vegetarians) had lower serum B12 than the less strict diet types (i.e. omnivores), with the exception of one study that only examined vitamin B12 nutriture in one diet type (vegetarians) [42].

A second study resulting from the EPIC-Oxford cohort study examining vitamin B12 nutriture in 689 men found that 52% of vegans (n=121) and 7% of vegetarians (n=16) were identified as deficient. In this sample, however, deficiency was defined as serum B12 less than 118 pmol/L[43]. Mean age for this study ranged from 42.8 years to 52.8 years old (ages were reported for diet groups only, not total the total sample). However, the vegan group had a significantly larger amount of young adults ranging from 20-39 years of age. Thus, it is likely that the deficiency prevalence would be slightly higher if 148 pmol/L were used to define deficiency. As this study only examined a male sample, these results cannot be extrapolated to a female population.

A third study examining vitamin B12 nutriture also found a higher prevalence of deficiency in vegetarians (n=53) and vegans (n=12), compared to omnivores (n=79) [44]. This study used the following vitamin B12 deficiency cut offs established by Herbert et al.: normal (MMA≤ 271 nmol/L and TCII≥ 35 pmol/L) stage I or II deficiency (MMA≤ 271 nmol/L and TCII<35 pmol/L), and stage III deficiency (MMA > 271 nmol/L and TCII < 35 pmol/L) [6]. The results of the study by Hermann et al. showed that 83% of vegans, 60% of vegetarians, and 3% of omnivores fell into the stage II deficiency category. Although there is a markedly higher prevalence of deficiency in the vegan sample from this study, the number of vegan subjects in this study is small (n=12) [44].

Vitamin B12 deficiency can present early on in life, with evidence of several cases of infant vitamin B12 deficiency in the literature [45]. Honzik et al. found that severe vitamin B12 deficiency in infants is predominantly influenced by low maternal B12 nutriture. Further, this study also showed that infants can still be at risk for deficiency, even if their mother's serum B12 concentration is considered normal. This is particularly the case when a mother's serum B12 concentration is on the low end of the 'normal' spectrum, as well as when a mother's breast milk contains inadequate B12 [45]. The majority of mothers in this study were not vegetarians, but rather had inadequate intestinal absorption of vitamin B12 [45]. Nonetheless, infants with vegetarian or vegan mother also tend to develop vitamin B12 deficiency early in life if maternal vitamin B12 nutriture is low [46]. In a study examining 27 infants with vitamin B12 deficiency symptoms in India who were exclusively breast-fed and all were born to vegetarian mothers, the authors found that 12 of these infants have low serum B12 [46]. Out of the other 15 infants, six did not have their serum B12 measured, and nine had normal serum

B12. However, seven of these nine infants had received supplemental vitamin B12 prior to enrolling in the study [46]. Twenty-two of the infants displaying symptoms of vitamin B12 deficiency were treated with supplemental B12. Although one subject was lost to follow-up, twenty of the infants treated with supplemental serum B12 had a dramatic reduction of symptoms [46]. Thus, although the symptoms of deficiency in an infantile population can be severe (such as failure to thrive, weaning difficulties, and developmental regression), supplementation is an important treatment tool and should be evaluated for use in at-risk populations prior to the diagnosis of maternal or infant deficiency.

Vitamin B12 Deficiency: Symptoms and Consequences

Symptoms of vitamin B12 deficiency generally fall into two categories: hematological and neurological. Hematological symptoms are thought to stem from the methylation cycle, where methylcobalamin acts as a coenzyme in the folate cycle in the conversion of homocysteine to methionine. Decreased vitamin B12 availability causes a decrease in methyleneTHF which results in a decreased conversion of deocyuridylate to thymidylate, leading to an accumulation of uracil. This excess uracil competes with thymine for incorporation into DNA coding for neucleoproteins, which ultimately leads to megaloblastic anemia [7]. The hematological presentation of vitamin B12 deficiency can include an increase in mean corpuscular volume (MCV), hypersegmentation of neutrophils, and macroovalocytes [8]. Ultimately, severe anemia can lead to symptoms such as fatigue, ankle edema, nocturia, dizziness, and orthostatic hypotension [8]. In addition, recent research has documented risk for decreased bone mineral density and osteoporosis in individuals with low vitamin B12 nutriture [47]. This prevalence of

deficiency is problematic because vitamin B12 is necessary for vital nervous system functions, including the synthesis of neurotransmitters and myelin sheaths. This myelination may be important for dexterity as the literature suggests possible connections between dexterity and myelination in adolescent-onset psychosis patients [10], dexterity and right cerebral white matter volume (white matter is bundles of myelinated axons in the brain [11]), and primary motor cortex volume in healthy older adults [12].

DNA Methylation

The lack of methylation capabilities due to the decreased functioning of methionine synthase in vitamin B12 deficiency and depletion can have substantial epigenetic consequences. Studies examining the effect of decreased vitamin B12 availability in utero, in both sheep and human models, have shown that the methylation patterns established during this time continue to affect the DNA methylation of said individual for the remainder of their life [20]. According to the "developmental origins of health and disease" hypothesis, this methylation is particularly important because prenatal DNA methylation can contribute to epigenetic regulation of disease risk later in life [48]. In spite of a large amount of research on DNA methylation, epigenetic regulation is still a relatively young field. Further, the extent to which B vitamins affect DNA methylation in adulthood is largely unknown [48]. In one recently conducted study (2015), investigators discovered an increased in DNA methylation in adults 65-75 years old after a two-year vitamin B12 supplement intervention. Some genes which displayed changes in methylation upon vitamin B12 supplementation include a tumor suppressor gene (DIRAS3), a gene associated with cancer metastasis (NODAL), and other genes association with embryonic development [48].

Nervous System Interactions

It is thought that one of the main reasons for the neurologic consequences in vitamin B12 deficiency is the interruption in myelination due to a buildup of methyl-malonic acid [20]. In addition, the methylation cycle is also thought to play a role due to a decrease in S-adenosyl methionine (SAM), which leads to a decrease in the methylation of proteins necessary for myelination as well as a decrease in white matter vacuolization [20]. Impaired DNA methylation, stemming from a decrease in methionine synthase activity, is also believed to affect the nervous system through a decrease in myelination and oligodendrocyte growth [7]. As myelin sheath is composed largely of lipids (80%), disturbances in fat metabolism can be especially problematic [20].

One of the more severe neurologic consequences of vitamin B12 deficiency are neural tube defects (NTDs). As the neural tube becomes fully closed by 28 days of gestation [20], ensuring adequate vitamin B12 nutriture for all women of reproductive age is essential to further decrease rates of neural tube defects at birth. Although it is commonly accepted that folate depletion drastically increases the risk for NTDs, Bell and Oakley estimated that only 75% of spina bifida and anencephaly can be prevented by folic acid fortification [49]. Inadequate maternal vitamin B12 nutriture possibly contributes to a large part of the remaining cases of NTDs, as a 2007 case-control study examining NTDs in 89 Canadian women (with 422 controls) found a nearly tripled risk of NTDs in the presence of low maternal B12 [50]. Further, Ray et al. suggests 34% of NTDs in Canada may be due to inadequate vitamin B12 [50]. A recent meta-analysis examining nine studies with cases of NTDs (n=567) and controls (n=1566) found the risk (OR) of developing NTDs was 2.31 if maternal B12 nutriture was inadequate [51]. When further

examining this risk, investigators discovered an elevated risk for NTDs due to low maternal B12 in developing countries (OR=5.07) than in developed countries (OR= 2.10) [51]. It is important to note that the authors suggest the strong possibility of publication bias in this area [51], meaning that many studies which discovered no association between maternal B12 nutriture and risk of NTDs were likely not published.

Clinically, however, the most recognized symptom of vitamin B12 deficiency is myelopathy, referred to by physicians as subacute combined degeneration. Early decreases in function associated with this degeneration include impaired vibration discernment, spastic paraparesis, and extensor plantar response [7]. In addition, optic [7], peripheral, and cranial neuropathies are also possible [7,52]. Other research has documented peripheral neuropathy, which includes a case study of a patient with onset of symptoms in the hands, where all limbs were also affected [7]. It is important to differentiate between B12 deficiency neuropathy and neuropathy associated with aging or other conditions. Thus a young adult population is ideal when studying this neuropathy.

Subclinical Vitamin B12 Deficiency

A large majority of current research on vitamin B12 focuses on the consequences of full deficiency, rather than subclinical deficiency or depletion. Concern for the consequences of subclinical deficiency, however, have recently been increasing due to the inefficacy of supplement therapy on nervous system symptoms in an elderly subject with low vitamin B12 nutriture [7]. According to Obeid and Herrmann, subclinical deficiency is a common problem today [53]. Although the frequency of this subclinical deficiency is not clearly defined, but it is estimated that subclinical deficiency is 10 times more common than clinical vitamin B12 deficiency. In addition, it is also believed that a

large number of individuals displaying subclinical deficiency may respond to supplements with a decrease in slight neurologic consequences [7]. Thus, identifying these patients and administering therapy before a more defined clinical deficiency rises is essential to prevent permanent neurological damage. There are several problems thought to contribute to the commonality of this subclinical deficiency, including a low RDA for vitamin B12 (some research has even suggested that it be increased to 6 μg/day) and an overestimation of hepatic storage abilities [53]. It is currently not known the exact serum concentration at which these subclinical will disappear, particularly in the older population [8]. Thus, due to this prevalence of subclinical deficiency, a normal serum B12 concentration would not necessary exclude vitamin B12 deficiency from the list of potential diagnosis. Clinicians will likely examine metabolites such as MMA and homocysteine to reveal a deficiency in patients with suspected vitamin B12 deficiency [8]. However, it is important to consider that other conditions may lead to an elevation in these metabolites, such as renal problems [8] or genetic disorders.

Dexterity

Dexterity is defined as the voluntary movements of the hands required to move or manipulate an object for a specific purpose [54]. Dexterity can be categorized into two different classifications, static or dynamic, based on grasping and manipulation requirements. Static dexterity does not require grasping or manipulation. An example of this is hitting something with a fist. Dynamic dexterity requires both grasping abilities and manipulative skill [55]. Dynamic dexterity can be further subdivided into two types of dexterity: manual and fine finger dexterity. Manual dexterity is the ability to move hands easily in a turning or placing motion. Fine finger dexterity is the ability to skillfully

move and handle an object with the fingers [55]. Fine finger dexterity requires much more precise motion and coordination than manual dexterity.

The decrease in myelination associated with vitamin B12 deficiency may be important for dexterity as the literature suggests possible connections between dexterity and myelination in adolescent-onset psychosis patients [10], dexterity and right cerebral white matter volume (white matter is bundles of myelinated axons in the brain [11]), and primary motor cortex volume in healthy older adults [12]. These negative impacts on hand functionality can decrease the quality of life as daily activities, personal medical care, or activities related to work are all affected [13]. Subtle changes in dexterity may reveal subclinical deficiency which, if identified early, can aid in the prevention of serious complications of vitamin B12 deficiency such as impaired vision, personality changes [7], psychosis, depression, mania [8], and dementia-like illness [9].

Purdue Pegboard Test

The Purdue Pegboard test is one of the most widely used tests to assess fine finger dexterity [55]. This test was originally designed to assess the dexterity aptitude of individuals interested in occupations requiring fine finger movements such as assembly positions or machine operators. This test provided a much cheaper way to assess employees than other tests of the time [56]. The reliability of the Purdue Pegboard test has been examined in several studies for one-trial administration. In a review of these studies, Buddenberg and Davis documented that the correlation coefficients of the subtests have ranged from 0.60 to 0.79 in both sexes, and up to 0.83 when only examining one sex [57]. In addition, investigators examining the test-retest reliability of one-trial administration with one week between tests of the Purdue Pegboard found ICCs

ranging from 0.37-0.70 for each of the Purdue Pegboard's assessments [57]. Perhaps a better method of analyzing this would be assessing dominant vs. non-dominant hand, as hand dominance likely plays a role in dexterity scores. The Purdue Pegboard is also administered in a 3-test bout, in which the reliability scores are predictably higher [56]. However, a "practice effect" exists with this test [57], which makes administering the test multiple times in one sitting questionable. Another important factor in test scores was the privacy of the test and knowledge of previous scores. Both of these cases increased the competitiveness of participants in one study [57], which may ultimately affect the validity of the results. Thus, the test should be conducted in a private room, and scores should not be shared with participants until after the completion of a study.

Figure 1: Purdue Pegboard with pegs for right hand dexterity test (top) and pieces for assembly test (bottom).

The Purdue Pegboard (pictured below) resembles a board game and is relatively simple and quick to administer. The board has two parallel lines of holes down the center of the board four cups at the top. The outside cups should contain half of the pins provided (~25). The center-right cup contains washers and the center-left cup contains sleeves. The test is administered in four parts: right hand, left hand, both hands, and assembly [56]. Prior to each

test, the subject is allowed to practice with 3-4 pieces before the time begins. In addition, the subject should sit at a comfortable table around 30 inches off of the floor. For both the right and left-hand tests, the subject is told to use only the respective hand to pick up one pin at a time and place it in the next consecutive hole as many times as possible during a thirty-second period. For this test, the score is the number of pins placed in the board [56]. This score should be directly related to vitamin B12 status. For the both hand test, the same procedure should be followed as the left and right test, but with both hands working simultaneously. For this test, the score is equal to the number of complete pairs placed in the board [56]. The scores of the right hand, left hand, and both hand tests are added up to create the right plus left plus both hand score, which is commonly used in research [56]. The assembly test (bottom picture above) is a more complex task and requires subjects to place a pin chosen from the right cup with the right hand, a washer over the pin placed with the left hand, a sleeve over the washer placed with the right hand, and a final washer over the sleeve placed with the left hand. For this task, the subject has 60 seconds to assemble as many towers as possible. For this task, the score is equal to the number of pieces placed on the board [56].

Functional Dexterity Test

The Functional Dexterity Test (FDT) is a commonly used assessment tool to evaluate manual dexterity in both adults and children [55].

Several studies have examined the reliability of this test and have found it is a valid measurement tool in populations such as individuals with musculoskeletal diseases, rheumatology conditions, and neuropathy. In addition, several studies have also found that the FDT is a valid tool to assess the association between dexterity and hand functions

such as handwriting and keyboard, as well as hand grip strength [55]. In addition, in a study conducted in healthy adults assessing the test-retest reliability of the FDT, investigators found an ICC of 0.94 for net time and 0.92 for total score [55]. In addition, investigators also found that demographic factors such as hand dominance and sex did not affect scores. Age, however, did affect scores for individuals older than 50 years old [55]. Thus, when dexterity is examined as an outcome in a research study, a younger population should be used to avoid decreases in dexterity associated with age from confounding results. Further, when examining dexterity in older adults, it is important to control for age in statistical analysis.

Figure 2: Functional Dexterity Test

Like the Purdue Pegboard, the Functional Dexterity Test (pictured below) resembles a board game and is very simple to administer. The test consists of a wooden board with 16 holes, each with a peg. The pegs are colored with different colors on the top and bottom. The objective of the test is to turn all of the pegs over and place them back in their respective holes as quickly as possible. The test must be conducted with each hand separately. The test is scored by the number of seconds it takes for the participant to complete turning each of the pegs over [55]. Time penalties are added for any errors in completing the test: 5 seconds for each time the participant touches or rests on the board for support and 10 seconds if the

participant drops a peg. The total time without penalties is the net time and represents hand dexterity speed. Adding the total time and the penalties gives the adjusted time, which represents the quality of the hand dexterity [55]. This score should be inversely related to vitamin B12 status. Unlike the Purdue Pegboard test, the FDT test is typically reported as the dominant or non-dominant hand [55], rather than left or right.

Balance

Balance can be measured by a multitude of analyses tools, including the force plate (FP) [58]. A laboratory grade force plate, such as the AMTI Accusway, is considered the "gold-standard" in measuring standing balance [59]. A force plate (such as the AMTI accusway) measures the force that a body exerts on the ground while in defined conditions (i.e. feet apart, feet together, one foot, etc.). In particular, a force plate measures the center of pressure (COP) of this ground reaction force, and the displacement in this force over a specified period of time [58].

According to Chaudhry et al., differences in COP displacements between two groups can be examined to compare balance. Other variables, such as area, velocity, and distance traveled, can also be used to characterize stability. However, there is still disagreement in the literature as to which variable produced by the FP most accurately portray one's balance capabilities [58]. Medio-lateral displacement is also an additional variable commonly examined in force plate studies. Interestingly, this variable has been shown to be associated with fall-risk in older adults [58].

Although research regarding the association between vitamin B12 and postural control is largely lacking, the relationship between balance and peripheral neuropathy is well documented in the literature [60]. Most (if not all) of this research is conducted on

subjects with particular health conditions such as diabetes and cancer. This change in balance is likely due to the decreased somatosensory feedback from the legs and feet, decreased proprioception, and decreased reaction time that occurs with peripheral neuropathy [60]. In severe cases, balance impairment due to neuropathy can lead to an increased fall risk [60,61]. One study examining balance in diabetic and non-diabetic women discovered significant differences between the mean amplitude of oscillation in both the anterior-posterior and medial-lateral direction, as well as the average speed of oscillation in the mediolateral direction [62]. A second study examining the differences in balance between a control group and breast cancer survivors suffering from chemotherapy-induced peripheral neuropathy found significant differences in center of pressure displacement. The authors of this study also discovered a correlation between the balance displacement and neuropathy symptoms [61]. A third recent study that examined the impact of long-term vitamin B12 supplementation on fall risk in older adults did not document a decrease in fall risk over time or a difference in fall risk between the intervention and control group. However the investigators did not examine any specific measures of balance in this study [63]. The findings of research studies such as these warrant investigation into the effects that vitamin B12 deficiency may have on neuropathy and precise measures of postural control in symptomatic and asymptomatic individuals.

Vibration Sensitivity

Decreases in vibration proprioception and vibration sense are physical symptoms of vitamin B12 deficiency related to changes in the spinal cord[52], specifically white matter deterioration in the posterior and lateral columns of the spinal cord. This can lead

to decreases in vibration sense because these columns contain sensory fibers which aid in vibration proprioception and, according to Simpson et. al, are especially sensitive to vitamin B12 deficiency [64]. In addition, deterioration of the peripheral nervous system can contribute to cutaneous sensory loss [52], such as loss in vibration sensitivity.

Several studies have documented the decrease in vibration proprioception due to vitamin B12 deficiency. One case study identifies a 57-year-old women with serum B12 concentrations within the normal range (albeit in the lower end) who presented with biochemical and neurological symptoms consistent with B12 deficiency, including impaired vibration sense [52]. A second study examining the prevalence of vitamin B12 and folate deficiency in the Indian state of Maharashtra documented decreased vibration sense in 5 subjects. However, of the subjects with serum B12 concentrations less than 200 pg/mL, 4 out of 4 subjects experiencing decreased vibration sense only experienced this in lower limbs. The method used to examine vibration sense was not mentioned by the authors of this study [65]. A third study examined the prevalence and clinical symptoms of vitamin B12 deficiency in psychiatric patients at a mental health intuition in Uganda. This study found that 12 patients had absent vibration sense, and that this was significantly correlated with low vitamin B12 (<240 pg/mL) in these patients. However, as these patients were residing in a mental health hospital, it is possible that they had other confounding neurological conditions at the time of the study. In addition, investigators did not disclose the method used to determine vibration sensation [66]. A fourth study examining the vibration sensitivity in individuals with a prior gastrectomy found that those who had low serum B12 concentrations had a significantly higher vibration threshold (inverse relationship) than those who had normal B12 concentrations.

Further, these individuals displayed a decrease in vibration thresholds (increased vibration sensitivity) when treated with vitamin B12 [67]. However, individuals with "inadequate therapy" or injections every month at most, did not display signs of improvement. The author of this study did mention, however, that although vibration sensitivity did improve, markers of myelopathy (such as Romberg's sign) remained unchanged [67].

Applications in Diabetes

The relationship between vitamin B12 and vibration sensitivity may have applications in the field of diabetes care and research. Vitamin B12 has been suggested as a treatment method for diabetic neuropathy (DN) [68]. Diabetic neuropathy is the primary cause of neuropathy in developed countries, and contributes to more diabetes-related hospitalizations than any other symptom of diabetes. Diabetic neuropathy can be extremely painful, with sensations ranging from tingling to burning, freezing, or stabbing pain [68].

A few studies have examined the effect of vitamin B12 supplementation on diabetic neuropathy, including one out of a diabetes clinic in Arak, Iran. This study examined the difference between intramuscular vitamin B12 injections (2000 μg) and oral nortriptyline pills, an antidepressant drug commonly prescribed to treat DN. In a single-blind trial with 100 subjects, the investigators discovered that the pain, tingling, and paresthesia symptoms decreased significantly more ($P<0.001$) in the intervention group than the standard of care group. However, other characteristics of diabetic neuropathy such as diminished vibration, position, or nerve conduction speed did not differ significantly across group or time [68]. Interestingly, the authors mentioned that

some of the individuals who displayed improvement with the administration of vitamin B12 had normal serum B12 pre-test results, illustrating the possibility of improvement in DN for all individuals, not just those who are vitamin B12 deficient [68].

As Metformin is commonly prescribed to aid in diabetes control, the diabetic population may be at a higher risk for developing vitamin B12 deficiency than the non-diabetic population [68]. Regardless, there are still no formal guidelines regarding vitamin B12 supplementation in diabetic patients. Some research suggests that multivitamin supplements do not contain enough cobalamin, as multivitamin use did not correct the inadequate vitamin B12 status in long-term Metformin users. Kibirige and Mwebaze suggest that a yearly 1000 ug dose of vitamin B12 may be enough to normalize serum B12, however they fail to identify a suggested method of supplementation [69]. Given the growing number of individuals diagnosed with diabetes, understanding the vitamin needs of these patients is of the utmost importance.

Vibratron II – Vibration Sensitivity Tester (Physitemp)

In the aforementioned study conducted by Talaei et al., a 128 Hz tuning fork was used to examine vibration sensitivity [68]. This method, however, is not ideal, as the frequency cannot be altered. More adaptable measures such as the Vibratron II are ideal for measuring vibration sensitivity because the vibration intensity can be decreased in small intervals to determine the vibration threshold, a reflection of the lowest vibration frequency sensed, of the patient or study subject.

The Vibratron II (pictured below) has been approved by the FDA for diagnostic use in determining cutaneous sensory loss [70]. In addition, according to a 2011 article, the Vibratron II is a useful tool in the detection of neuropathy in type 1 diabetic patients [71].

Figure 3: Vibratron II – Vibration Sensitivity Tester

Prior research has examined the use of the Physitemp Vibratron II in examining vibration thresholds and neuropathy related to diabetes [72-75], chemotherapy agents [76], and multiple sclerosis [77,78] with positive results. However, no research has yet been conducted using the Vibratron II to examine the relationship between vibration threshold and serum B12 concentrations.

The Vibratron II is composed of two testing blocks, labeled A and B, with two small rods on the top of each testing block. Both testing blocks are attached to a central control device that governs the vibration intensity of the rods on the testing blocks, as well as the testing block selection. One switch on the control unit determines the test block selection, while a second dummy switch is present to prevent subject knowledge of switching between testing blocks. The vibration sensitivity test can be conducted at either high-resolution (0-6.5 vibration units, or 0-20 microns) or low-resolution (6.5-19.9 vibration units, or 20-200 microns) frequencies. High-resolution frequencies vibrate at a much faster rate and are more difficult to detect than low-resolution frequencies.

Before the test begins, participants are instructed to use only the pointer finger on their dominant hand, be consistent with pressure throughout the test (enough pressure to begin to turn the fingernail yellow), to touch each rod for only one second, to touch each rod only once, and to touch A then B consecutively. The test begins at a predetermined

vibration intensity at which most participants are likely to sense. The participants are allowed 5 attempts at this intensity to become comfortable with the test before the data collection begins. If the participant correctly identifies the vibrating rod at all 5 switches, then testing will proceed. Throughout the trial, the test administrator randomly switches between testing blocks, while using the dummy switch to ensure a switch is flipped after each attempt. When a participant correctly identifies the vibrating rod, the vibration intensity for the next attempt is decreased by 10%. If a participant incorrectly identifies the vibrating rod, that intensity is repeated. After two incorrect identifications, the intensity is increased by 10% and the test will continue. If an incorrect identification is followed by a correct identification, one more attempt is made at that intensity. To eliminate the likelihood of a streak of correct guesses, all intensities below 0.7 vibration units (VU) will be repeated twice. After five incorrect identifications, the test is completed. The vibration units of the five errors and the five lowest scores are compiled, with the highest and lowest of these numbers thrown out to eliminate outliers. The vibration threshold in VU is the average of these eight scores. This vibration threshold can also be converted to microns.

Supplementation

Currently, research examining vitamin B12 supplementation is sparse, as much of the data is subsidiary information reported in studies examining other factors. However, much of the data available shows that deficiency rates in supplement users are still much higher than expected [1]. However, in one of these studies, the supplement users were removed from statistical analysis due to the small number of individuals in this group [41]. In a larger second study, which consisted of 689 adult male participants of differing

dietary habits, only 19% of vegetarians and vegans, compared to 4% of omnivores, reported regularly taking a vitamin B12 containing supplement [43]. Of the supplement users, 89% of vegetarians and 63% of vegans consumed 1.5 μg of vitamin B12 per day. This is surprising as many of those taking supplements were still well below adequate consumption. Interestingly, however, the investigators of this study did not find a significant difference between the serum B12 levels of supplement users and non-users in each dietary group [43]. Although the authors acknowledge that this could be due to a reporting error, this discrepancy also suggests a problem with efficacy or absorption of vitamin B12 supplements [43]. Some studies reveal that vitamin B12 supplements should be administered in mega-doses, even up to 100 times the current RDA [1].

Oral vitamin B12 supplementation is typically recommended for individuals at risk for deficiency, such as vegetarians or those who have had a gastrectomy. In cases of deficiency, however, clinicians recommend a different supplementation regimen [8]. Typically, 1 mg/day of intramuscular vitamin B12 injections is followed by 1 mg/week for one month, then 1 mg/month for the duration of the patient's life. According to Briani et al., oral doses of 1-2 mg/day will suffice for individuals who prefer to avoid maintenance injections. However, this is typically only prescribed after body stores have been normalized by initial injections [8].

If a prescribed supplement regimen is rigorously adhered to, symptoms of vitamin B12 deficiency begin to resolve soon after supplementation begins, with all abnormal hematologic symptoms resolving within 8 weeks [8]. The improvement in neurologic symptoms after treatment, however, is much more varied. Although treatment typically leads to some improvement in neurologic symptoms, some residual effects may still

remain. However, the literature generally supports the idea that early treatment yields the best results [8].

Although there have been many vitamin B12 supplementation studies conducted, few examine the effects of supplementation in asymptomatic individuals. One recent study examined the effect of on intramuscular vitamin B12 injection on nerve conduction speed in deficient, yet asymptomatic, elderly adults [79]. Investigators measured nerve conduction speed of the sural nerve (calf region) on both legs, as well as the median nerve (forearm) on the right arm before and after a B-vitamin complex containing 10mg of cobalamin. The results of this study indicated a significant improvement in sural nerve conduction speed, which the investigators attribute to the vitamin B12 supplement. Although improvements in the sural nerve were clear, changes in the median nerve were less not well-established [79]. However, as both niacin and vitamin B6 were also included in the injection, it is possible that these vitamins also played a role in the improvement of nerve conduction speed. Still, this study exhibits the need for further investigation of vitamin B12 supplementation on nerve conduction speed and other measures of peripheral nerve function. Further, Brito et al. suggests that peripheral nerve conduction measurements may be the best method of detecting sub-clinical B12 deficiency [79].

CHAPTER 3

METHODS

Participants

Healthy vegetarian adults were recruited for this study through social media outreach, e-mail LISTSERVs, vegetarian support groups, and flyers posted at ASU and vegan restaurants. Study advertising materials included the purpose of the study, age and dietary habits of participants needed, location for the study (Arizona State University downtown Phoenix campus), general time availabilities, and compensation information. Inclusion criteria included a willingness to travel to the Downtown ASU Nutrition laboratory for study visits. Other qualifying criteria included age, diet habits and medication use. This study was submitted to and approved by the Institutional Review Board Committee at Arizona State University. All participants reviewed an informed consent statement prior to beginning the screening survey, and signed a detailed written consent form prior to official enrollment in the study.

Participants were 19-40 years of age and represented a young adult population, which was the focus of this investigation. Because B12 is only present in animal products [6], vegetarians and vegans are more likely than omnivores to be deficient in vitamin B12. All participants adhered to their respective vegetarian or vegan diet for at least 3 years, a criteria based on the knowledge that there is an increased prevalence of vitamin B12 deficiency for vegans and vegetarians who had adhered to their diet for 2-4 years [1]. In addition, the liver is known to store up to 3mg of vitamin B12, which can sustain the body above a state of depletion for 3-5 years [20].

Since certain medications are known to affect vitamin B12 metabolism, volunteers were asked to report if they regularly used the following medications: proton pump inhibotors (i.e. Nexium®, Prevacid®, Prilosec®) [80], anticonvulsants [81], potassium chloride [82], colchicine, certain antibiotics (neomycin), biguanides (Metformin) [8], and nitrous oxide [83]. Volunteers with medical conditions that would prevent safe completion of all aspects of this study, as well as those with nervous system disorders, were also excluded from participation. These conditions include severe arthritis, severe carpel tunnel syndrome, hemophilia, and neurological movement disorders (i.e. Parkinsons, multiple sclerosis). In addition, participants diagnosed with pernicious anemia, an autoimmune disease that prevents B12 absorption, were excluded.

Study Design

Screening: An online survey was used to screen participants. Prior to beginning the survey, participants were prompted to review an informed consent statement. Individuals were informed that, by continuing with the survey, they agreed to participate in the screening process. This initial screening provided information regarding the respondents' contact information, age, dietary habits (omnivore, lacto-ovo vegetarian, or vegan), duration of dietary habits, medical conditions that prevent participation in the study, medication use, ability to commute to the study location, and scheduling availability. Respondents also were asked to acknowledge that, if they choose to participate in the study, they must arrive having fasted from food and drink (with the exception of water) for 8 hours prior to the scheduled study visit. Selection options for this statement included "I Agree and am medically capable of fasting for 8 hours", "I do not agree (select this option if you are medically unable to fast for 8 hours)", and "I

choose not to answer". If the survey respondents met the criteria to be included in this study, they were contacted by e-mail (or phone if necessary) to schedule a study visit.

Study Visit #1. Participants were sent e-mail reminders 24 hours before the study visit regarding the location and time of their study visit, as well as a reminder to fast for 8 hours prior to meeting with investigators. The study visit began with investigators providing the participants with an informed consent document. After participants read and signed the informed consent document, participants were assigned a subject number for de-identification of the data to protect participant privacy. Height and weight were then measured. After investigators had confirmed that participants fasted for 8 hours, a 10 ml blood sample was collected via venous blood draw by a trained phlebotomist. Upon completion of the blood draw, snacks were provided to participants while they rested and completed a health history questionnaire and food frequency questionnaire. The health history questionnaire asked information regarding demographic characteristics (i.e. sex, age, race, ethnicity, education background, household income bracket, etc.), diet history, supplement history, and incidence of major disease. After participants completed the forms and finished their snacks, participants were asked to confirm a lack of hypoglycemic symptoms such as feeling "shaky, lightheaded, or hungry". Next, participants engaged in four functional tests. Directions were read to participants while investigators demonstrated each test (see literature review and appendices B-E for more detailed instructions on the tests discussed below in steps 1-4).

1. Balance: Participants were asked to stand on the AMTI Accusway force plate with their feet at a 30˚ angle. They were instructed to stare at a fixed point on the

wall (about eye level) while staying as still as possible for one minute. Their center of pressure sway was analyzed during this period.

2. Vibration Sensitivity: Participants were instructed to touch two rods, in sequence, and determine which rod was vibrating. Investigators randomly switched which rods were vibrating, while decreasing the vibration intensity throughout the test.
3. Purdue Pegboard test: Participants were asked to place small metal pins into a line of holes in sequence, as quickly as possible in 30 seconds. Participants will complete the pegboard test 3 times each with their right hand, left hand, and both hands. Participants will then complete an assembly test, where they created small towers with metal washers and sleeves, 3 times with both hands for 60 seconds each.
4. Functional dexterity test: Participants were instructed to turn over all wooden pegs on a board as quickly as possible with their dominant hand. The test was then repeated on their non-dominant hand. Net time (time to turn over all pegs) and the total score (net time + time penalties) will be recorded for this test.

Upon completion of the functional testing, participants were debriefed and offered compensation for their time ($5 Target® gift cards).

Study Visit #2*:* Twenty-nine of the initial participants for visit #1 were invited back to participate in a follow-up study. Twenty-five of these participants enrolled in the follow-up study. After serum samples were analyzed, these participants were stratified by age, gender, BMI, and vitamin B12 status and randomly assigned to either the intervention or control group, and After consent forms were signed, participants were given bags containing 56 pills (500 mcg Nature Made™ B12 or placebo of 250mg 25%

Acetic Acid Nature's Life™ apple cider vinegar pills), a supplement consumption log, and a username and password for ASA24 (an online, 3-day diet record log). Investigators were blinded to the contents of the bag. Participants were instructed to consume one supplement each day for eight weeks. Participants were scheduled for study visit #3 at this study visit. Throughout the 8 weeks, emails were sent to remind participants take their supplements and complete their 3-day diet record. An 8 week time line was selected based on the normalization of blood count when introducing B12 supplements to deficient patients [7,8].

The vitamin B12 dosage selected for this intervention trial (1000 mg/d) is safe based on the fact that there is no upper limit of vitamin B12 [84]. One study examining the effects of B12 on homocysteine levels as well as incidence of acute myocardial infarction in individuals 30-85 years of age found no adverse side effects of participants ingesting 0.4 mg of B12 daily (167x the DRI) for 40 months [85]. In addition, a second study examining the effects of vitamin B12 supplementation homocysteine concentrations and incidence of stroke and coronary artery disease in individuals >55 years of age found no adverse side effects of participants ingesting 1.0 mg of B12 daily (2400x the DRI) for 5 years [86].

Study Visit #3*:* Participants were sent e-mail reminders 24 hours before the study visit regarding the location and time of their study visit, as well as a reminder to fast for 8 hours prior to meeting with investigators. Investigators collected supplement bags with remaining supplements and supplement consumption logs. Height and weight were also measured. The same protocol for study visit #1 was repeated for the blood draw, vibration sensitivity, balance, and dexterity tests. Upon completion of the functional

testing, participants were debriefed and offered compensation for their time ($20 Target® gift cards).

Lab Assays. All laboratory analysis took place in the School of Nutrition and Health Promotion Laboratory in the Arizona Biomedical Complex building on the Arizona State University downtown Phoenix campus. Assays were overseen by the Senior Research Nurse and Radiology Technologist. After the blood samples were collected, they were centrifuged in order to separate the plasma that was stored in the laboratory freezers until analyses. The remaining components of the blood were properly discarded. Duplicate assays were performed for each serum sample using a B12/Folate Radioasay kit (SimulTRAC-SNB Vitamin B12/Folate RIA Kit; MP Biomedicals; Santa Ana, CA) and gamma radiation detection (Wallac Wizard Gamma Counter 1470; Perkin Elmer; Waltham, Massachusetts). Each kit permitted the analysis of 32 duplicate samples over a 4 hour time period. This assay provided cobalamin, holo-transcobalamin II (TCII), and folate serum concentration information. Individual participant sample pairs with a high coefficient of variation (>10%) were re-run in order to prevent laboratory error.

Serum vitamin B12 and folate concentrations are determined by a radioassay technique that measures the proportion of both the endogenous vitamin and the exogenous radiolabeled vitamin bound to high affinity, specific binding compounds. In this assay, BIORAD Lyphocheck® human serum assay standards and controls are used to develop the standard curve. To examine total serum vitamin B12 concentrations, 200 μL of vortexed serum, standards, and controls are added to empty12x75 mm polypropylene tubes (see Appendix F). A mixture of the radiolabeled vitamins (B12 and

folate) and a Dithiothreitol solution is added to the serum samples. This mixture is then vortexed and incubated at room temperature under a cover of aluminum foil for 15 minutes. Next, 100 μL of extracting reagent is added to the mixture, which is again vortexed and incubated at room temperature under a cover of aluminum foil for 10 minutes. The combination of the dithiothreitol and the alkaline pH of the extracting reagent functions to degrades all proteins bound to vitamin B12 and folate. Thus, the resulting mixture contains free endogenous and exogenous vitamin B12 and folate. Blank reagent (1000 μL) is then added to one sample (two tubes) to act as a control for the addition of the binding reagent. Next, 1000 μL of the binding reagent is added to all standard, control, and reference tubes. This binding agent must be vigorously shaken before transferring to the sample tubes, as it tends to clump and stick to the walls of its container. The binding reagent contains both a purified intrinsic factor (B12 binder) and a purified folate binder. The mixture is then vortexed and incubated at room temperature for 1 hour under aluminum foil. After 1 hour, the mixture is centrifuged at 4°C for 10 minutes at 3000 rpm. Next, the tubes are removed from the centrifuge and handled gently so as not to disturb the solid pellet at the bottom of the tube. The supernatant fluid, which contains excess serum compounds and unbound radiolabeled vitamins, is removed by decanting each tube and wiping excess fluid from the rims of the tubes. Finally, the samples are run through the gamma counter, which provides printouts detailing the standard curves and values for each sample's total serum B12 and folate. As each binder has a higher affinity for the endogenous forms of the vitamins, the amount of radioactive isotopes in the final pellet is inversely proportional to the serum concentration of the vitamin B12 and folate.

Transcobalamin II serum concentration is evaluated using the technique developed by Das et al [87]. In this assay, 3 grams of microfine silica (silicon dioxide) from Sigma-Aldrich is combined with 20 mL of distilled water. This mixture is placed on a stir plate and thoroughly mixed. The edges of the container are continually scraped with a glass rod to prevent the silica from clumping. While stirring, 100 μL of the silica slurry is combined with 500 μL of serum. The slurry is the incubated at room temperature for 10 minutes under a cover of aluminum foil. Next, the mixtures are centrifuged at 5000 X g for 10 minutes. After carefully removing the samples from the centrifuge, the supernatant fluid is transferred to separate tubes. These samples are then analyzed for B12 using the above radioassay technique. As the silica binds to holo-transcobalamin II, the supernatant liquid in this portion of the assay will represent transcobalamin I and III. Thus, to determine the serum concentration of TCII, the results of this assay are subtracted from the results of the total serum B12 assay.

Statistical Analysis

The sample size was calculated using 80% power and an α of 0.05. Based on the below table, the ideal sample size for this study is 45 subjects, accounting for 20% attrition to ensure a final sample size of 36 (18 subjects per group). The sample size was calculated based on the primary outcome variables of dexterity and vibration sensitivity. Sample size calculations based on median motor nerve conduction velocity are included below, as this outcome variable was originally intended to be included as a part of this study. Measuring nerve conduction velocity in a non-invasive and cost-efficient manner, however, was deemed unfeasible. Therefore, nerve conduction velocity* was removed from this study. The standard deviation for vibration sensitivity was taken from the

population average documented in the Physitemp Vibratron-II Operating Manual. The minimal detectable differences were calculated using the difference between the population average (0.7) in the Physitemp Vibratron-II Operating Manual and the average vibration threshold (1.05) documented in Martin et al.'s study examining 1,177 diabetic adults.

Sample Size Calculations

Outcome Variable	Standard Deviation	Source	Minimal Detectable Difference	Source	Sample Size
Purdue Pegboard: Right-hand dexterity	1.33	Preliminary Data (2nd-year study)	1.3	Telles et al. [88]	36
Vibration Sensitivity	0.4	Physitemp Vibratron-II Operating Manual	.305	Physitemp Vibratron-II Operating Manual, Martin et al. [89]	44
Median motor nerve conduction velocity*	4.2	Sahin et al. [90]	5.7	Sahin et al. [90]	20

Table 3. Sample size calculations utilizing dexterity and vibration sensitivity. Median motor nerve conduction was used to calculated sample size for the study before it was removed as an outcome variable.

Statistical analysis was conducted using the Statistical Package for Social Sciences (SPSS 21 for Windows, 2010, Chicago IL) software program. Normality was assessed, and non-normal data was transformed. Demographic information was compiled and presented using data from the initial screening survey, as well as the health history questionnaire. Data is reported as mean ± SD.

Cross Sectional

Pearson's correlations were conducted for all parametric and normalized data. Spearman's correlations were conducted for non-parametric data points. Partial correlations, controlling for folate, were also conducted for all data points. For non-parametric variables, the most parametric transformation was used in partial correlation analyses.

Outcome variables were compared between several groups: vegetarians and vegans, short-term and long-term diet adherence, supplement users and non-users, and combined supplement users and B12/B-complex supplement users. For normally distributed variables, group differences were calculated using independent t-tests. For non-parametric variables, group differences were calculated using Mann-Whitney U tests.

Intervention

Two-way repeated measures ANOVAs were used to test mean differences for each of the outcome variables (vibration sensitivity, balance variables, functional dexterity, Purdue pegboard dexterity) by main effects of time (pre, post) and group (intervention, control), as well as an interaction effect (time x group). General linear models were also used to test mean differences for the main and interaction effects after adjustment for covariates.

CHAPTER 4

RESULTS

Cross-Sectional Data Analysis

Of the 351 individuals who responded to a screening survey, 105 individuals qualified for this study. Individuals were screened out of the study if they were an omnivore, had not adhered to a vegetarian diet for at least three years, had a history of cancer or diabetes, were not between the ages of 18 and 40, were pregnant or breastfeeding, had false nails that extended past the fingertips, or were unable/unwilling to provide blood samples and commute to study sites. Thirty-eight subjects were ultimately enrolled in the study. The majority of qualified participants, who qualified but did not enroll, were not enrolled due to lack of e-mail responses or scheduling availability.

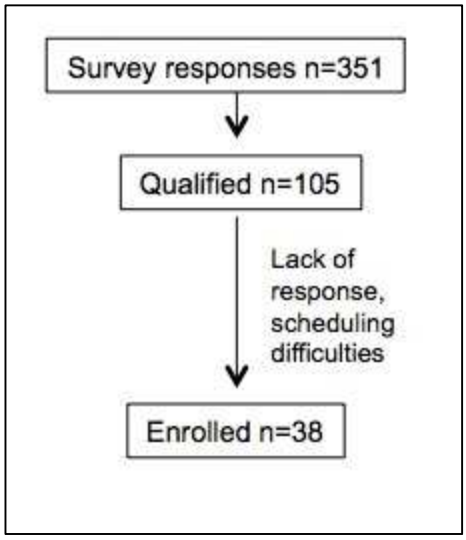

Figure 4. Participant recruitment and retention for the cross-sectional portion of thus study.

Demographic characteristics of the 38 enrolled participants are shown in Table 4 and Table 5 below. The majority of participants were Caucasian females who had been

vegan for more than five years. The average age of participants is 28.4 y. The average BMI is 22.0 kg/m^2, falling into the normal weight BMI category.

Table 4: Demographic frequencies in the total study population.

Variable	n (%)
Total	38
Gender	
Male	9 (23.6)
Female	29 (76.3)
Ethnicity	
Native American	0
African-American	0 (0)
Caucasian	32 (84.2)
Hispanic	1 (2.6)
Asian	5 (13.2)
Other	0
Diet	
Vegan	23 (60.5)
Lacto-Ovo Vegetarian	15 (39.5)
Diet Length	
2-3 years	1 (2.6)
3-5 years	15 (39.5)
More than 5 years	22 (57.9)
Activity	
Not active	0 (0)
Somewhat active	13 (34.2)
Active	15 (39.5)
Very Active	10 (26.3)

Table 5: Demographic characteristics (Mean ± SD) of the 38 subjects enrolled in the cross-sectional portion of the study.

Variable	Mean	SD
Age (y)	28.4	5.5
BMI (kg/m^2)	22.0	2.7
Physical Activity (MET minutes/week)	62.6	6.5
Alcohol consumption (drinks per week)	1.4	2.3

Shapiro-Wilk normality tests of outcome variables revealed normal distribution for the following variables: some balance markers (x-axis maximum velocity, average velocity, path length), all Purdue Pegboard variables (right-hand average, left-hand average, both hand average, right/left/both hand average, assembly average), one functional dexterity test variable (right hand raw), and transcobalamin II. The following variables were abnormally distributed: some balance markers (y-axis maximum velocity, area effective, 95% area), vibration threshold (in vibration units and microns), most functional dexterity test variables (right hand adjusted, left hand raw, and left hand adjusted), folate, and vitamin B12. Functional dexterity test raw scores is the time (seconds) to complete each test without any time penalties added. A (*) in the tables below indicates non-parametric distribution. For these values, non-parametric tests were used to assess outcomes.

Table 6: Normality Testing of Major Outcome Variables

Outcome	Variable	Significance	Transformation	Significance
Balance	Vy Max	0.028*	Inverse	0.000*
	Vx Max	0.091		
	V average	0.119		
	Path Length	0.119		
	Area Effective	0.000*	Logarithm	0.878
	Area 95	0.000*	Logarithm	0.656
Vibration Threshold	VT (Vibration Units)	0.047*	Square Root	0.016*
	VT (Microns)	0.000*	Square Root	0.035*
Fine Dexterity *Purdue Pegboard*	Right Hand	0.828		
	Left Hand	0.615		
	Both Hands	0.494		
	Right/Left/Both Hands	0.707		
	Assembly	0.724		
Manual Dexterity *Functional Dexterity Test*	Right Hand Raw	0.199		
	Right Hand Adjusted	0.005*	Logarithm	0.385
	Left Hand Raw	0.016*	Logarithm	0.200
	Left Hand Adjusted	0.006*	Logarithm	0.104

Biochemical Data	Folate	0.005*	Logarithm	0.864
	B12	0.000*	Logarithm	0.661
	TCII	0.090		

With logarithmic transformations, the distribution of the following variables was normalized: area effective, area 95, FDT right hand adjusted, FDT left hand raw, FDT left hand adjusted, folate, and B12. Vibration threshold (in both vibration units and microns) and Vy max were not able to be normalized with logarithmic, inverse, and square root transformations.

The relationship between serum B12 and TCII displayed a strong correlation (R=0.667) (figure 5).

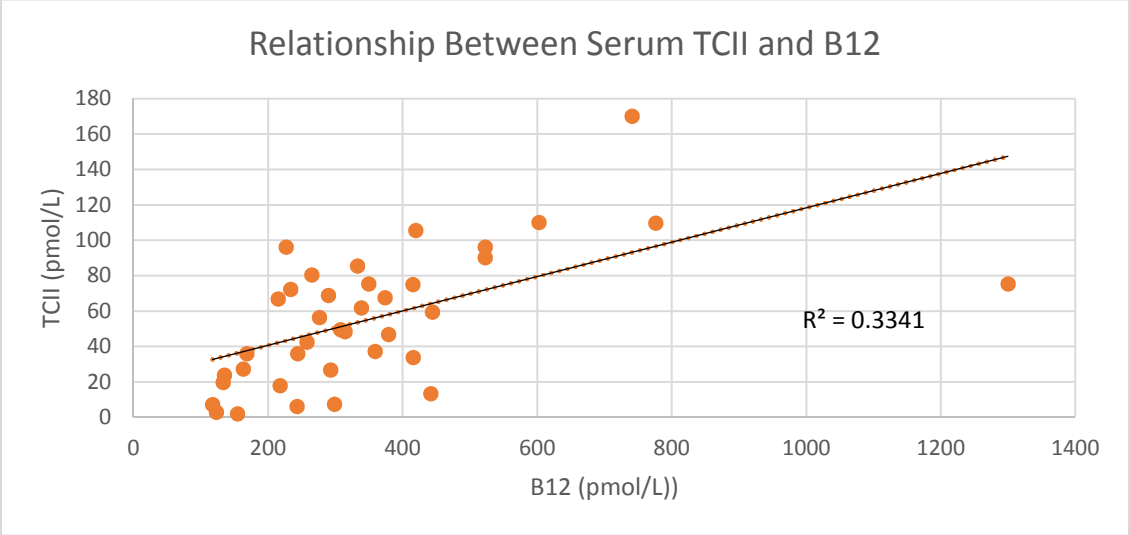

Figure 5: Association between serum TCII and B12. R values obtained from parametric correlations with TCII and log transformed B12.

The balance, dexterity, and vibration sensitivity mean results are displayed below. Table 7 displays group differences by diet, table 8 displays group differences by diet length, table 9 displays group differences between supplement users and non-users, and

Table 10 displays group differences between those taking combined supplements (B12 combined with other nutrients) and stand-alone B12 or B-complex supplements. Lower values are desired for the following variables: Vy max, Vx max, V average, path length, area effective, area 95, VT, right hand adjusted, and left hand adjusted. Higher values are desired for the right hand, left hand, both hands, right/left/both hands, assembly, folate, B12 and TCII. Confounder analysis revealed that gender is a confounder for Vx max and the Functional Dexterity Test right hand adjusted outcomes. In addition, BMI is a confounder for the Purdue Pegboard assembly score. In the tables below, higher values are desired for the variables marked with an (**). Lower values are desired for all other variables.

Table 7. Mean Difference for Outcome Measures between Vegans and Vegetarians

Variable	Total (n=38)		Vegan (n=23)		Vegetarian (n=15)		*p*
	Mean	SD	Mean	SD	Mean	SD	
Vy Max (in/sec)	-3.19	1.42	-3.31	1.60	-3.00	1.12	0.643
Vx Max (in/sec)	3.01	1.30	3.05	1.14	2.96	1.53	0.584
V average (in/sec)	0.939	0.335	0.935	0.353	0.945	0.318	0.930
Path Length (in)	56.4	20.1	56.1	4.5	56.7	19.1	0.929
Area Effective (in^2)	1.18	1.27	1.12	1.08	1.27	1.55	0.920
Area 95 (in^2)	2.59	2.30	2.60	2.44	2.59	2.16	0.894
VT (Microns)	0.394	0.306	0.424	0.366	0.351	0.190	0.881
Right Hand (pins per 30 sec)**	15.4	1.6	15.4	1.4	15.5	1.8	0.786
Left Hand (pins per 30 sec)**	14.6	1.6	14.7	1.5	14.3	1.9	0.517
Both Hands (pins per 30 sec)**	12.4	1.5	12.5	1.4	12.3	1.6	0.693
Right/Left/Both Hands (pins per 30 sec)**	42.4	4.1	42.6	3.6	42.1	4.9	0.766
Assembly (pieces per 60 sec)**	28.9	3.9	28.7	3.7	29.3	4.1	0.226

Right Hand Adjusted (seconds)	23.3	6.0	23.9	6.3	22.4	5.5	0.220
Left Hand Adjusted (seconds.)	26.9	9.1	26.4	10.0	27.5	8.0	0.462
Folate (nmol/L)**	42.3	19.0	40.4	17.6	45.0	21.2	0.444
B12 (pmol/L)**	354.9	224.4	378.7	261.9	320.0	156.4	0.653
TCII (pmol/L)**	55.5	37.7	63.3	39.6	44.4	32.7	0.128

There was no significant difference in outcome variables between vegetarians and vegans. Of the 23 vegan participants, ten had adhered to their diet for more than 5 years. In addition, 14 of the 23 vegan participants reported taking B12 containing supplements. Of these 14 vegan supplement users, 7 reported taking B12 or B-complex supplements. Of the 15 vegetarian participants, 12 had adhered to their diet for more than 5 years. In addition, 7 of the 15 vegetarian participants reported taking B12 containing supplements. Of these 7 vegetarian supplement users, 3 reported taking B12 or B-complex supplements. Of the vegan participants, 14 supplemented with B12 containing supplements and 9 did not supplement with B12 containing supplements. Of the vegetarian participants, 7 supplemented with B12 containing supplements and 8 did not supplement with B12 containing supplements.

Table 8. Mean Difference for Outcome Measures by Length of Diet Adherence

Variable	Total (n=38)		3-5 years (n=16)		> 5 years (n=22)		*p*
	Mean	SD	Mean	SD	Mean	SD	
Vy Max (in/sec)	-3.19	1.42	-2.95	1.34	-3.35	1.48	0.307
Vx Max (in/sec)	3.01	1.30	2.72	1.23	3.22	1.33	0.194
V average (in/sec)	0.939	0.335	0.925	0.338	0.949	0.340	0.834
Path Length (in)	56.4	20.1	55.5	20.3	57.0	20.4	0.834
Area Effective (in^2)	1.18	1.27	1.14	1.20	1.21	1.34	0.795
Area 95 (in^2)	2.59	2.30	2.48	2.66	2.67	2.07	0.483
VT (Microns)	0.394	0.306	0.508	0.409	0.317	0.183	0.203

Right Hand (pins per 30 sec)**	15.4	1.6	16.0	1.3	15.1	1.7	0.082
Left Hand (pins per 30 sec)**	14.6	1.6	15.5	1.6	13.9	1.3	0.003
Both Hands (pins per 30 sec)**	12.4	1.5	13.4	1.0	11.7	1.3	0.000
Right/Left/Both Hands (pins per 30 sec)**	42.4	4.1	44.9	3.0	40.6	3.9	0.001
Assembly (pieces per 60 sec)**	28.9	3.9	30.7	3.2	27.7	3.9	0.035
Right Hand Adjusted (seconds)	23.3	6.0	22.3	4.9	24.0	6.6	0.268
Left Hand Adjusted (seconds.)	26.8	9.1	23.4	6.2	29.2	10.2	0.042
Folate (nmol/L)**	42.3	19.0	46.7	22.6	39.2	16.0	0.329
B12 (pmol/L)**	354.9	224.4	390.0	276.6	331.0	184.0	0.498
TCII (pmol/L)**	55.5	37.7	51.3	32.4	58.4	41.4	0.583

There was a significant difference between the short-term (3-5 years) and long-term diet adherence groups for all Purdue Pegboard outcomes measuring fine dexterity, except one. Individuals in the long-term diet adherence group scored significantly less on left hand dexterity ($p=0.003$), both hand dexterity ($p<0.001$), right/left/both hand combined dexterity ($p=0.001$), and the assembly test ($p=0.010$). The difference between groups for right hand dexterity is trending towards significance ($p=0.082$). There was no difference between the long-term and short-term diet adherence groups for any other balance, vibration sensitivity, or manual dexterity variables. Although there was a significant difference in fine dexterity outcomes between the diet adherence groups, there was no significant difference in vitamin B12 or TCII values between the two groups. However, the mean serum B12 for those who had adhered to their diet for more than five

years (331.0 ± 184.0) was lower than the mean serum B12 for those who had adhered to their diet for 3-5 years (390.0 ± 276.6). The TCII values for both diet adherence groups were similar, but the mean TCII for the short-term diet adherence group (51.3 ± 32.4) was lower than the mean TCII for the long-term adherence group (58.4 ± 41.4).

Table 9. Mean Difference for Outcome Measures Between Participants Taking and Not Taking Vitamin B12 Containing Supplements

Variable	Total (n=38)		Taking (n=21)		Not Taking (n=17)		*p*
	Mean	SD	Mean	SD	Mean	SD	
Vy Max (in/sec)	-3.19	1.42	-3.08	0.946	-3.31	1.85	0.927
Vx Max (in/sec)	3.01	1.30	2.89	1.02	3.16	1.59	0.484
V average (in/sec)	0.939	0.335	0.960	0.326	0.915	0.354	0.685
Path Length (in)	56.4	20.1	57.6	19.6	54.9	21.2	0.685
Area Effective (in^2)	1.18	1.27	1.04	0.920	1.35	1.61	0.709
Area 95 (in^2)	2.59	2.30	2.37	1.95	2.85	2.69	0.805
VT (Microns)	0.394	0.306	0.344	0.227	0.454	0.377	0.246
Right Hand (pins per 30 sec)**	15.4	1.6	15.6	1.4	15.2	1.7	0.374
Left Hand (pins per 30 sec)**	14.6	1.6	14.9	1.7	14.2	1.5	0.265
Both Hands (pins per 30 sec)**	12.4	1.5	12.6	1.5	12.2	1.5	0.397
Right/Left/Both Hands (pins per 30 sec)**	42.4	4.1	43.1	4.2	41.6	4.0	0.276
Assembly (pieces per 60 sec)**	28.9	3.9	29.8	3.9	27.9	3.6	0.091
Right Hand Adjusted (seconds)	23.3	6.0	24.0	7.1	22.4	4.4	0.752
Left Hand Adjusted (seconds.)	26.9	9.1	23.3	7.1	31.0	9.7	0.005
Folate (nmol/L)**	42.3	19.0	47.8	20.8	42.8	17.2	0.740
B12 (pmol/L)**	354.9	224.4	429.3	268.9	267.4	111.4	0.017
TCII (pmol/L)**	55.5	37.7	67.1	39.1	41.9	31.9	0.041

The B12 containing supplement users had significantly better (p=0.005) left-handed manual dexterity than those not taking B12 containing supplements. In addition, participants who reported consuming B12 containing supplements had significantly higher serum B12 (429.3 ± 268.9, p=0.017) and TCII (67.1 ± 39.1, p=0.041) than those who did not report consuming B12 containing supplements (267.4 ± 111.4, 41.9 ± 31.9 respectively).

When comparing the participants who received their supplemental vitamin B12 from multivitamin supplements and those who received their supplemental vitamin B12 from stand alone B12 or B-complex vitamins, there was a significant difference in left hand adjusted manual dexterity (p=0.014, favoring stand alone supplement users), left hand fine dexterity (p=0.042, favoring multivitamin users), and average velocity, path length, and area effective (p=0.037, p=0.037, p=0.027 respectively; favoring stand-alone supplement users).

Among those consuming B12 containing supplements, ten participants reported consuming their supplemented B12 within a combination multivitamin or powder. Eleven participants reported consuming their supplemental B12 as stand-alone B12 or B-complex supplements. The participants who reported taking stand-alone B12 or B-complex supplements had significantly better balance outcomes scores for average velocity (p=0.037), path length (0.037), and area effective (0.027) than the participants who reported taking combined B12 supplements. Stand-alone B12 supplement users also had significantly worse left hand Purdue Pegboard scores (p=0.042) and left-hand manual dexterity scores (p=0.014) than combined B12 supplement users. Finally, there was no significant difference in serum B12, TCII, or folate concentrations between stand-alone

and combined B12 users. Although not significant, the mean serum B12 of stand-alone vitamin B12 supplement users was slightly higher (446.4 ± 362.1 pmol/L) than that of the combined supplement users (412.2 ± 144.5 pmol/L).

According to Table 11 below, left hand adjusted manual dexterity was moderately correlated with serum B12 concentration (R = -0.351, p=0.031). Figure 6 shows the relationship between non-transformed serum B12 and Functional Dexterity Left Hand Adjusted scores. Figure 7 shows the relationship between B12 and Functional Dexterity Left Hand Adjusted scores using logarithmic transformations for both values to attain normal distribution. The average Y and X axis velocities, as well as area 95, displayed moderate and weak correlations with serum B12 that were trending toward significance (R = 0.314, p=0.058; R = -0.286, p=0.086; R = -0.296, p=0.075 respectively). The Purdue Pegboard assembly test scores were also moderately correlated (R = 0.317) with serum B12, with a p-value trending towards significance (p=0.052). TCII was not significantly correlated with any outcome variables. Controlling for serum folate concentrations did not alter the significance of any correlation. Controlling for hand dominance strengthened the correlation between serum B12 and left-hand manual dexterity (p=0.029, R=-0.360). In addition, controlling for hand dominance also revealed significance between serum B12 and Purdue Pegboard assembly scores (p=0.027, R=0.363).

Table 11. Correlations between biochemical values and outcome variables.

	B12		TCII		Folate		B12		TCII	
Controlling for							Folate		Folate	
	R	p	R	p	R	p	R	p	R	p
Vy Max (in/sec)	0.314	0.058	0.101	0.550	0.181	0.283	0.289	0.088	0.088	0.608
Vx Max (in/sec)	-0.216	0.207	-0.001	0.997	-0.055	0.751	-0.209	0.227	0.003	0.988
V average (in/sec)	-0.149	0.378	0.022	0.897	0.050	0.769	-0.163	0.343	0.018	0.917
Path Length (in)	-0.149	0.377	0.022	0.898	0.050	0.769	-0.163	0.343	0.018	0.918
Area Effective (in^2)	-0.247	0.140	-0.082	0.629	-0.040	0.814	-0.245	0.151	-0.079	0.646
Area 95 (in^2)	-0.296	0.075	-0.097	0.570	-0.081	0.635	-0.286	0.090	-0.091	0.599
VT (Microns)	0.135	0.419	0.116	0.488	0.052	0.758	0.128	0.452	0.112	0.508
Right Hand (pins per 30 sec)**	0.058	0.731	-0.216	0.193	0.084	0.615	0.044	0.798	-0.225	0.188
Left Hand (pins per 30 sec)**	0.039	0.818	-0.174	0.297	0.076	0.648	0.019	0.910	-0.182	0.288
Both Hands (pins per 30 sec)**	0.126	0.452	-0.172	0.301	0.295	0.072	0.073	0.672	-0.206	0.228
Right/Left/Both Hands (pins per 30 sec)**	0.083	0.622	-0.213	0.199	0.169	0.310	0.050	0.771	-0.231	0.175
Assembly (pieces per 60 sec)**	0.237	0.157	-0.132	0.437	0.322	0.052	0.188	0.273	-0.166	0.334
Right Hand Adjusted (seconds)	0.143	0.404	0.203	0.235	-0.222	0.193	0.189	0.276	0.222	0.199
Left Hand Adjusted (seconds)	-0.351	0.031	-0.172	0.302	-0.226	0.173	-0.343	0.041	-0.163	0.341

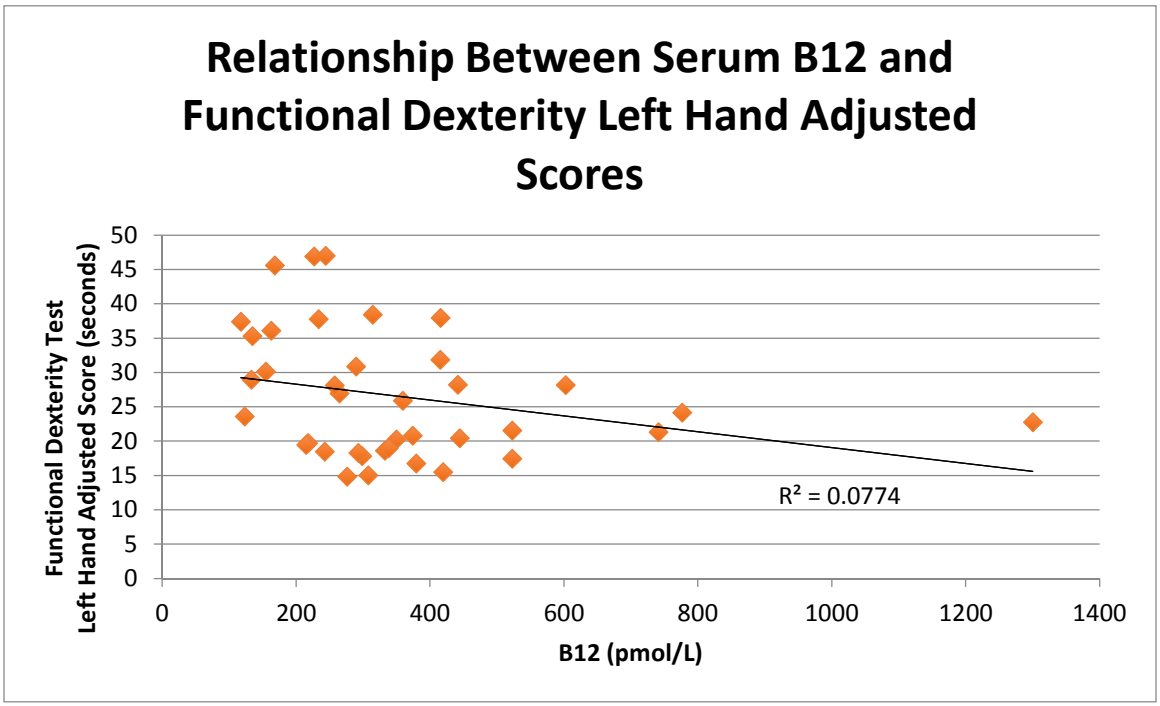

Figure 6: Association between serum B12 and left-hand adjusted scores from the Functional Dexterity Test.

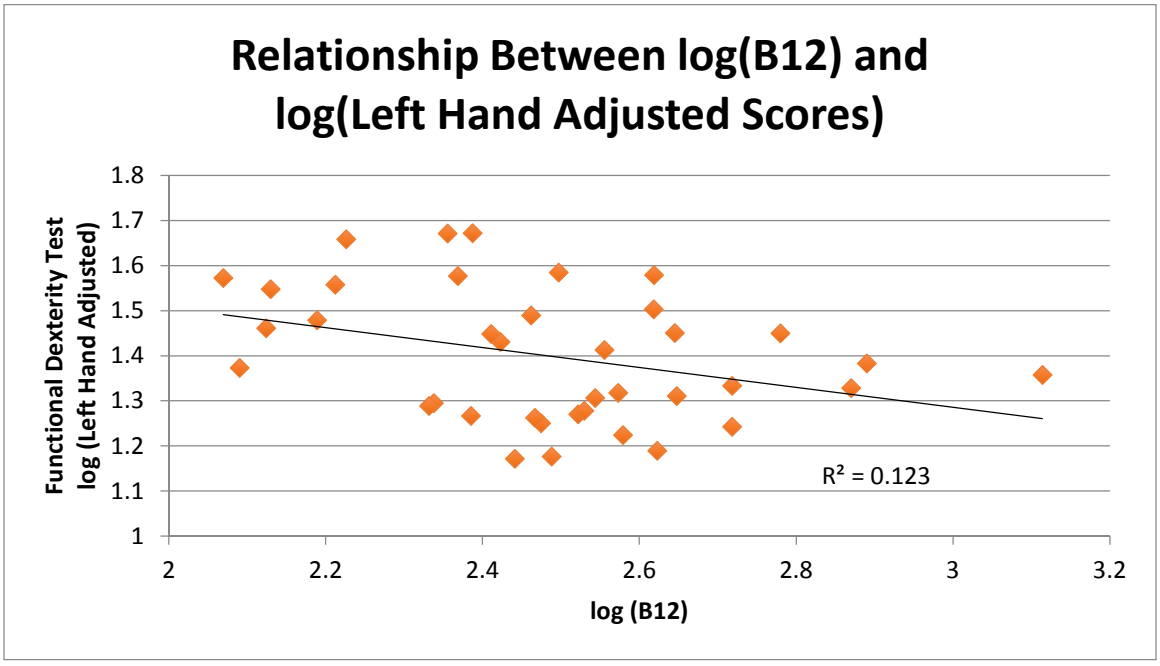

Figure 7: Association between log(serum B12) and log(left hand adjusted score) from the Functional Dexterity Test.

Finally, we explored the differences between two groups of participants clustered using the 50th percentile for serum vitamin B12: 303 pmol/L. When comparing the low serum B12 and high serum B12 groups, the low B12 group required significantly more time to complete the left hand Functional Dexterity Test ($p = 0.034$). In addition, the difference between the low and high B12 groups was trending toward significance for the Purdue Pegboard Assembly test and the area95 balance outcome variable ($p=0.057$, $p=0.057$). However, when the area95 variable was transformed using square root, the difference between the low and the high B12 groups became significant ($p=0.030$).

Intervention Data Analysis

Twenty-eight participants were qualified and randomly assigned for the intervention portion of this study (figure 8). Four participants did not complete the initial visit. Six participants dropped from the study during the intervention, the majority of whom dropped due to scheduling conflicts. Ultimately, 18 subjects completed the intervention study: 8 in the placebo group and 10 in the vitamin B12 supplement group.

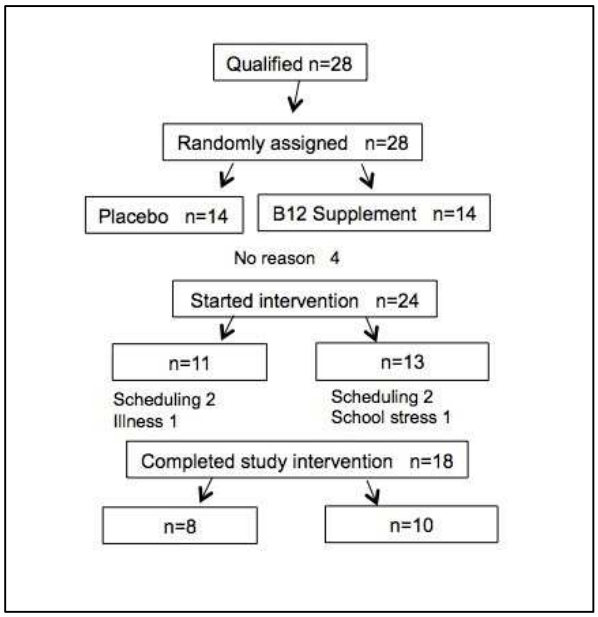

Figure 8. Participant recruitment and retention for the intervention portion of thus study.

Demographic characteristics of the 18 participants enrolled in the intervention portion of the study are shown below in Tables 12 & 13. The majority of participants were Caucasian females, eight being vegetarian and ten being vegan. The average age of the intervention participants was 27 years. The average BMI of participants was 22.3 kg/m^2, falling into the normal weight category. The average supplement adherence for all subjects is 2 pills missed for both the intervention and placebo groups. All participants in the intervention portion of this study were right-handed, so no adjustments for hand dominance were needed.

Table 12: Demographics characteristics for Intervention and Control Participants

Variable	Number (%)			
	Total (n=18)	Intervention (n=10)	Placebo (n=8)	
Gender				
Male	4 (22.2)	2 (20.0)	2 (25.0)	
Female	14 (77.8)	8 (80.0)	6 (75.0)	
Ethnicity				
Native American	0	0	0	
African-American	0	0	0	
Caucasian	15 (83.3)	8 (80.0)	7 (87.5)	
Hispanic	1 (5.6)	1 (10.0)	0	
Asian	2 (11.1)	1 (10.0)	1 (12.5)	
Other	0	0	0	
Diet				
Vegan	10 (55.6)	7 (70.0)	3 (37.5)	
Lacto-Ovo Vegetarian	8 (44.4)	3 (30.0)	5 (62.5)	
Diet Length				
3-5 years	8 (80.0)	6 (60.0)	2 (25.0)	
More than 5 years	10 (55.6)	4 (40.0)	6 (75.0)	
Activity				
Not active	0	0	0	
Somewhat active	8 (44.4)	3 (30.0)	5 (62.5)	
Active	5 (27.8)	5 (50.0)	0	

Very Active	5 (27.8)	2 (20.0)	3 (37.5)	
Supplementation				
B12 Containing Supplements	9 (50)	6 (60)	4 (50)	

Table 13. Demographic Characteristics of the 18 subjects enrolled in the intervention portion of the study.

	Total (n=18)		INTV (n=10)		Placebo (n=8)	
Variable	Mean	SD	Mean	SD	Mean	SD
Age	27.1	5.6	26.0	5.4	28.50	5.8
BMI	22.3	2.9	22.0	2.5	22.8	3.4
Physical Activity (METs weekly)	71.6	47.0	80.8	50.6	60.0	42.4
Alcohol consumption (drinks per week)	1.3	2.5	0.8	1.6	1.9	3.4

Shapiro-Wilk normality tests of outcome variables revealed normal distribution for the following variables: some balance markers (y-axis maximum velocity, average velocity, path length), all Purdue Pegboard variables, all functional dexterity test variables, folate, and transcobalamin II. The following variables were abnormally distributed: some balance markers (x-axis maximum velocity, area effective, 95% area), vibration threshold (in vibration units and microns), and vitamin B12.

Table 14. Normality Testing of Major Outcome Variables in the Intervention Participants

Outcome	Variable	Significance	Transformation	Significance
Balance	Vy Max	0.935		
	Vx Max	0.010*	Logarithm	0.455
	V average	0.059		
	Path Length	0.059		
	Area Effective	0.000*	Logarithm	0.263
	Area 95	0.001*	Logarithm	0.319
Vibration Threshold	VT (Vibration Units)	0.014*	Logarithm	0.596
	VT (Microns)	0.000*	Logarithm	0.596

Fine Dexterity *Purdue Pegboard*	Right Hand	0.927		
	Left Hand	0.938		
	Both Hands	0.967		
	Right/Left/Both Hands	0.839		
	Assembly	0.926		
Manual Dexterity *Functional Dexterity Test*	Right Hand Raw	0.415		
	Right Hand Adjusted	0.083		
	Left Hand Raw	0.444		
	Left Hand Adjusted	0.123		
Biochemical Data	Folate	0.421		
	B12	0.000*	Logarithm	0.136
	TCII	0.334		

Confounder analysis reveals physical activity to be a confounder for average velocity, path length, area effective, and area 95. This analysis also revealed age to be a confounder for functional dexterity test right-hand adjusted scores. Finally, TCII is confounded by diet type. Due to measurement errors, subject 22's balance data at time 2 was removed from the analysis. Subject 27s serum B12 level is an outlier, as it was more than three standard deviations away from the mean.

Table 15: Time*Group Significance and Effect Size for Outcome Variables

Outcome	Variable	Intervention						Placebo						p	pη2
		Pre		Post		Δ		Pre		Post		Δ			
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
Balance	Vy Max	-2.54	0.830	-2.62	2.88	0.25	2.96	-2.95	0.86	-3.88	2.25	-0.94	2.38	0.556	0.025
	Vx Max	2.99	1.58	2.54	3.09	-0.45	3.23	2.62	0.915	3.69	1.83	1.08	2.18	0.113	0.170
	V average	0.832	0.215	0.862	0.667	-0.02	0.65	0.978	0.302	1.104	0.599	0.13	0.72	0.859	0.003
	Path Length	49.92	12.92	15.90	10.73	-31.80	14.29	58.72	18.09	17.79	8.56	-40.9	18.66	0.209	0.118
	Area Effective	1.47	2.041	0.202	0.172	-1.19	1.92	0.971	0.510	0.216	0.166	-0.70	0.60	0.568	0.026
	Area 95	2.69	2.92	0.466	0.433	-2.09	2.75	2.46	1.54	0.552	0.376	-1.91	1.66	0.732	0.009
Vibration Threshold	VT (Vibration Units)	0.864	0.424	0.786	0.234	-0.120	0.466	0.824	0.202	0.707	0.107	-0.12	0.184	0.751	0.007
	VT (Microns)	0.453	0.507	0.333	0.173	-0.167	0.492	0.357	0.163	0.255	0.080	-0.1	0.150	0.640	0.015
Fine Dexterity *Purdue Pegboard*	Right Hand**	15.4	1.3	16.2	1.4	0.8	1.1	15.9	1.6	16.4	1.4	0.6	0.6	0.470	0.035
	Left Hand**	14.9	1.0	15.0	1.4	0.1	0.7	14.3	2.1	14.9	2.2	0.7	0.6	0.081	0.189
	Both Hands**	12.7	1.3	12.8	1.6	0.2	0.7	12.3	1.7	12.4	1.8	0.2	0.6	0.857	0.002
	Right/Left/Both Hands**	42.9	3.4	44.0	4.3	1.1	1.7	42.5	4.9	43.7	5.2	1.6	1.0	0.832	0.003
	Assembly**	29.3	3.0	30.8	3.9	1.5	2.5	28.4	5.7	29.6	2.8	2.0	3.7	0.837	0.003

Table 15: Time*Group Significance and Effect Size for Outcome Variables

Outcome	Variable	Intervention						Placebo						p	pη2
		Pre		Post		Δ		Pre		Post		Δ			
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
Manual Dexterity *Functional Dexterity Test*	Right Hand Raw	21.9	3.2	21.3	3.7	-0.1	3.1	22.1	6.8	21.0	4.0	-1.9	3.4	0.745	0.007
	Right Hand Adjusted	21.9	3.2	23.5	7.6	2.4	5.9	23.4	8.6	23.5	7.1	-0.4	11.0	0.468	0.038
	Left Hand Raw	22.8	5.5	21.3	3.8	-0.9	3.3	24.5	6.9	24.5	6.4	0.1	2.4	0.180	0.116
	Left Hand Adjusted	26.2	6.7	22.4	5.3	-2.9	4.9	29.5	12.9	26.4	7.5	-3.4	8.2	0.807	0.004
Biochemical Data	Folate**	42.2	20.9	37.1	22.2	-5.0	14.4	52.2	22.8	37.9	15.2	-14.3	17.8	0.239	0.085
	B12**	418.1	339.4	572.9	466.4	154.8	186.8	336.0	184.7	315.1	147.8	-20.6	137.9	0.042	0.234
	TCII**	57.5	36.2	123.6	127.7	66.1	126.6	50.2	35.9	42.6	36.4	-7.8	65.4	0.155	0.122

Table 16: Change data for outcome variables and effect size for this change data reported as Cohen's D

Outcome	Variable	Intervention		Control		Pooled SD	Cohen's D
		Δ Mean	Δ SD	Δ Mean	Δ SD		
Balance	Vy Max	0.025	2.96	-0.936	2.383	2.687	0.358
	Vx Max	-0.448	3.233	1.077	2.185	2.759	0.553
	V average	-0.025	0.653	0.0125	0.721	0.688	0.055
	Path Length	-31.78	14.29	-40.93	18.66	16.619	0.551
	Area Effective	-1.190	1.920	-0.699	0.597	1.422	0.345
	Area 95	-2.09	2.75	-1.91	1.66	2.271	0.079
Vibration Threshold	VT (Vibration Units)	-0.120	0.466	-0.117	0.184	0.354	0.008
	VT (Microns)	-0.167	0.492	-0.109	0.140	0.362	0.160
Fine Dexterity *Purdue Pegboard*	Right Hand**	0.8	1.1	0.7	0.6	0.886	0.113
	Left Hand**	0.1	0.7	0.7	0.6	0.652	0.920
	Both Hands**	0.2	0.7	0.1	0.7	0.700	0.143
	Right/Left/Both Hands**	1.1	1.7	1.2	1.4	1.557	0.064
	Assembly**	1.5	2.5	1.2	4.1	3.396	0.088
Manual Dexterity *Functional Dexterity Test*	Right Hand Raw	-0.1	3.1	-1.1	3.8	3.468	0.288
	Right Hand Adjusted	2.4	5.9	0.1	10.3	8.393	0.274
	Left Hand Raw	-0.9	3.3	0.0	2.2	2.804	0.321
	Left Hand Adjusted	-2.9	4.9	-3.1	7.7	6.447	0.036
Biochemical Data	Folate**	-5.0	14.4	-14.3	17.8	16.190	0.574
	B12**	154.8	186.8	-20.6	137.9	164.181	1.068
	TCII**	66.1	126.6	-7.8	65.4	100.759	0.733

Table 15 and Table 16 both display effect size calculations. Table 15 contains effect size using partial eta squared, while table 16 contains effect size using Cohen's D. Unless otherwise noted, all effective size mentioned hereafter is referring to partial eta squared. With the exception of vitamin B12, there were no significant time*group differences for any outcome test variables (Table 15). There was a significant time*group difference in serum vitamin B12 ($p=0.039$). In the intervention group, the serum B12 increased significantly after 8 weeks ($p=0.017$ including the outlier, $p=0.032$ excluding the outlier). In the placebo group, the serum B12 did not change over time ($p=0.609$). The mean change in B12 over time increased by 31% in the intervention group and decreased by 8% in the placebo group (graph 4). Graphs 5 and 6 display the individual variation within the intervention and placebo groups. The serum B12 outlier is marked by an (*). When the time*group interaction is analyzed for serum B12 with the outlier removed, the significance is lost ($p=0.061$) and the effect size decreases to 0.214. Upon removal of the outlier, the change in mean serum B12 over time for the intervention group decreased to 109.8 pmol/L. When analyzing time*group interactions only for participants whose baseline serum B12 concentration was less than 303 pmol/L (intervention $n=5$, $\Delta=101.3$ pmol/L; placebo $n=4$, $\Delta=-20.1$ pmol/L), the time*group interaction was not significant, but had a partial effect size of 0.387.

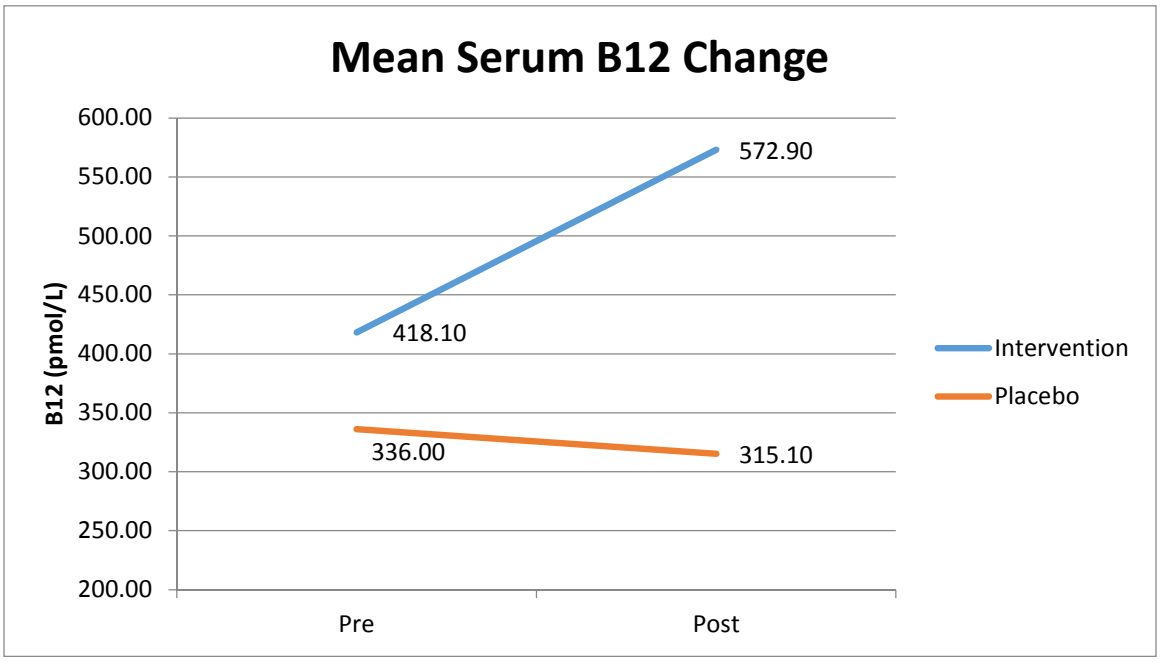

Figure 9: Change in mean serum B12 over time by the intervention and placebo groups.

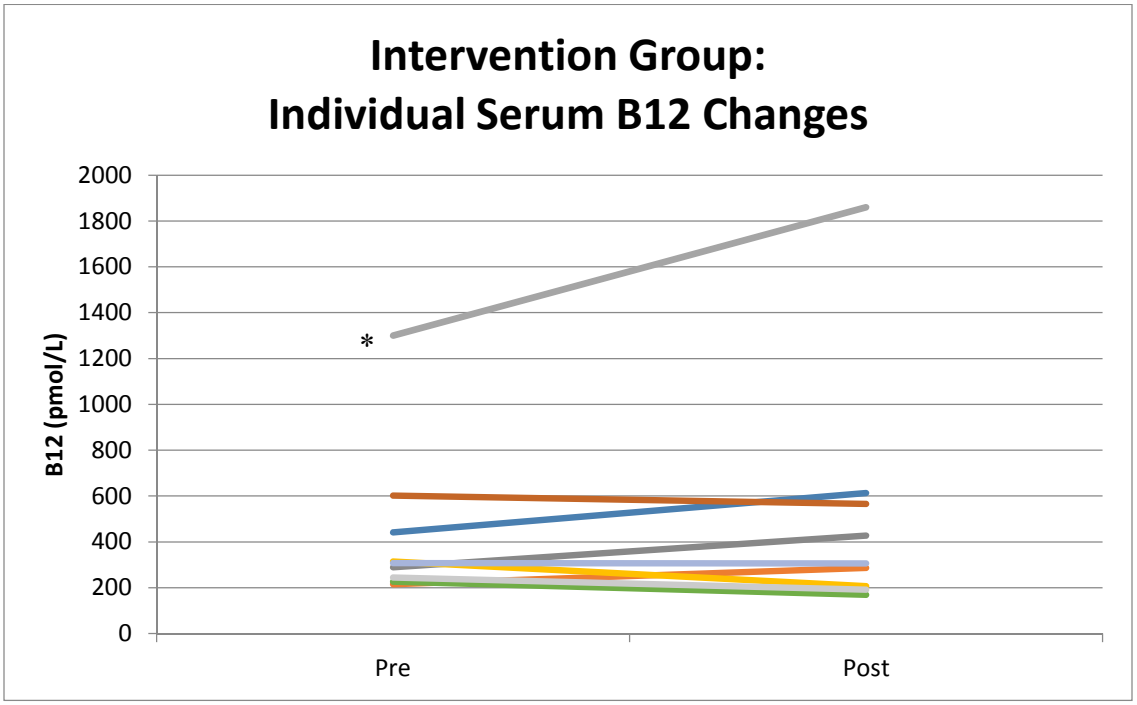

Figure 10: Changes in serum B12 over time for intervention group participants.
*Indicates an outlier.

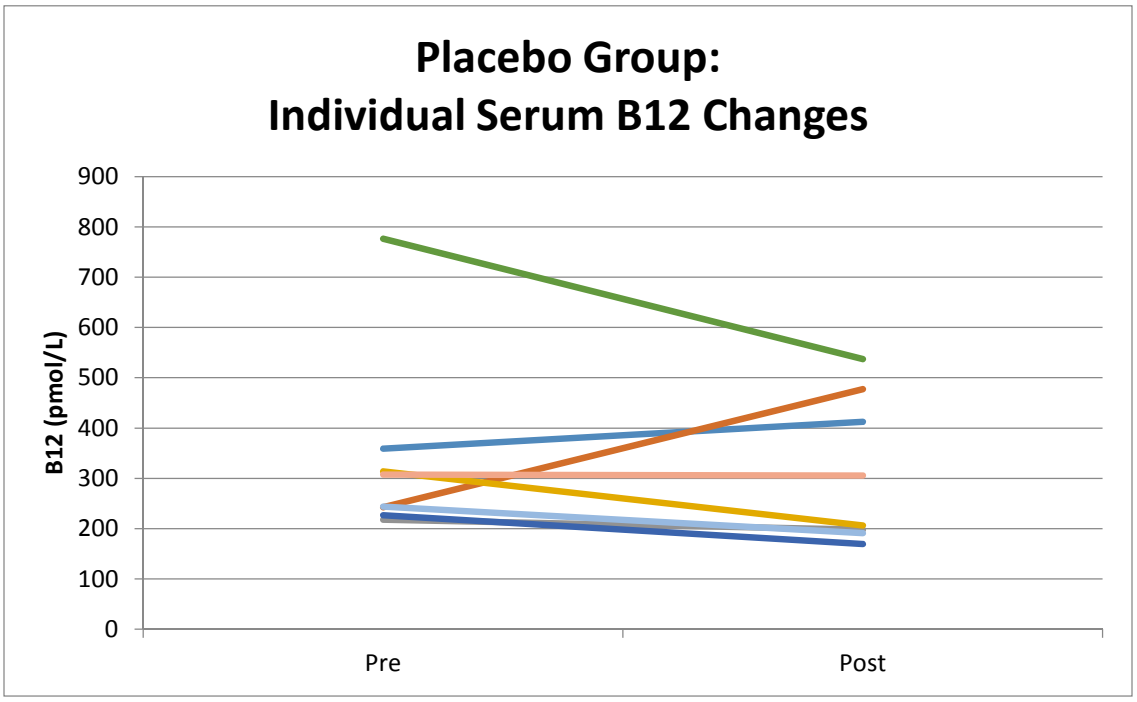

Figure 11: Changes in serum B12 over time for placebo group participants.

The mean change in TCII increased by 115% over time in the intervention group and decreased by 15% over time in the placebo group (figure 9). TCII serum concentrations did not increase significantly in the intervention group over time (p=0.133 including the outlier, p=0.128 excluding the outlier). In addition, the TCII serum concentration did not change over time (p=0.746). Graphs 8 and 9 display the individual variation within the intervention and placebo groups. When the time*group interaction is analyzed for serum TCII with the serum B12 outlier participant removed, the effect size decreases to 0.101 and remains non-significant (p=0.213). Upon removal of the outlier, the change in mean serum TCII over time for the intervention group decreased to 29.1 pmol/L.

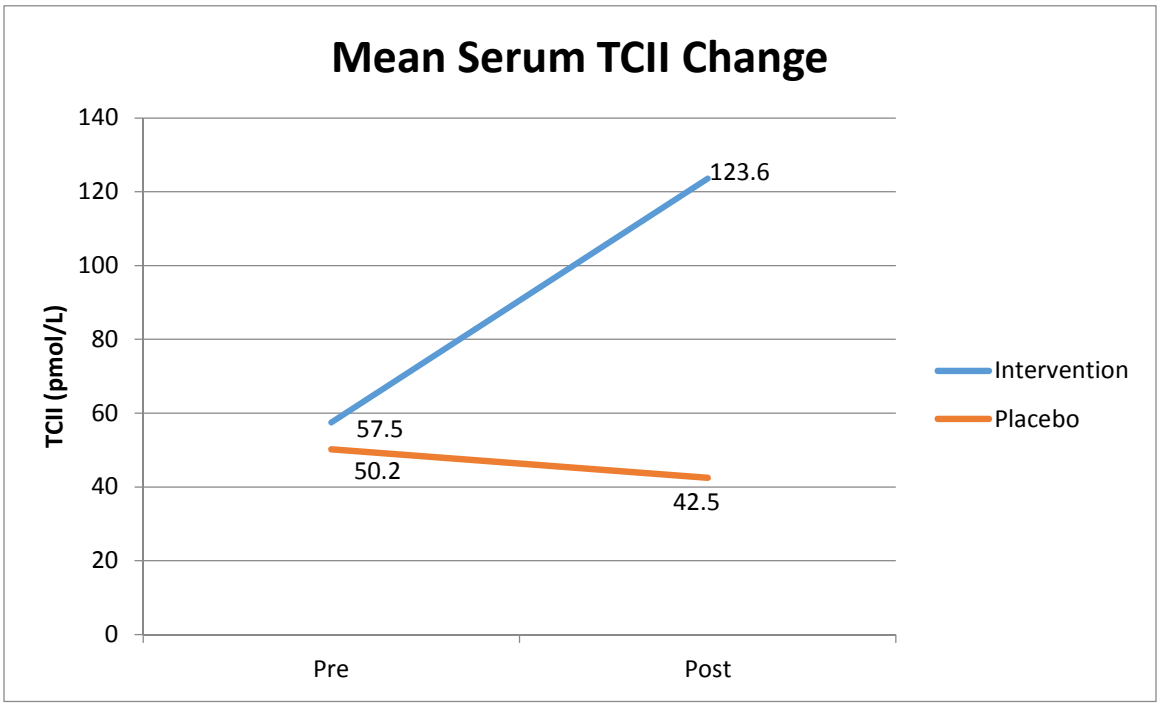

Figure 12: Change in mean serum TCII over time for the intervention and placebo groups.

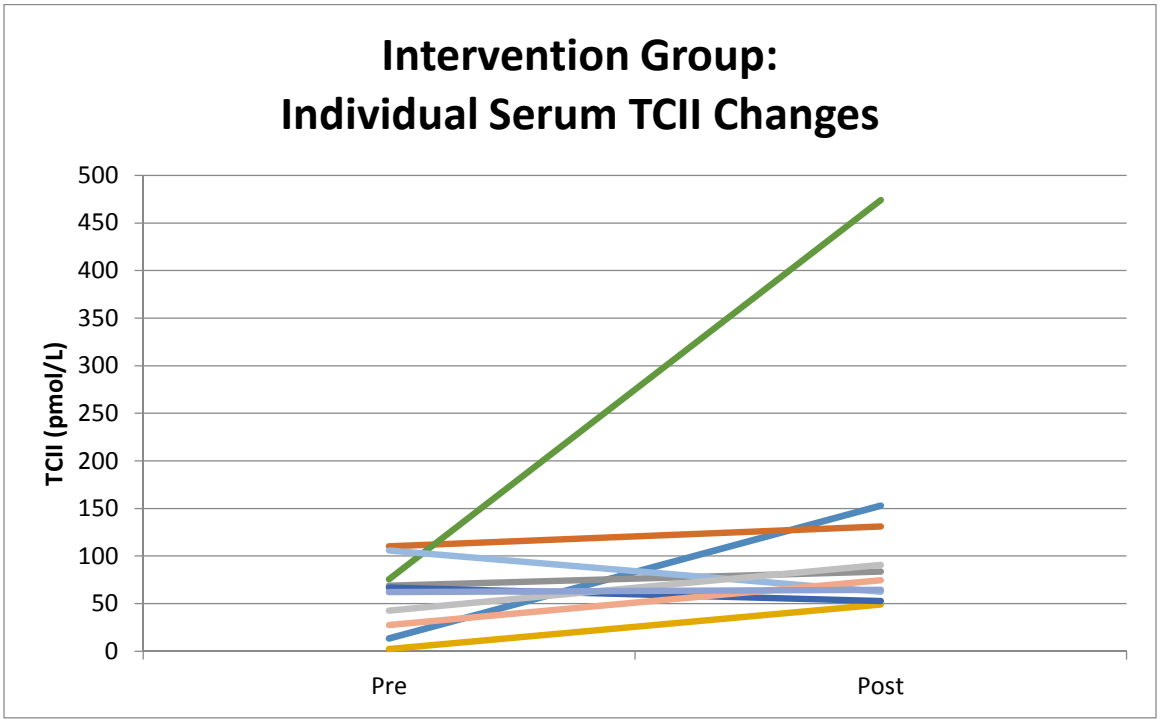

Figure 13: Changes in serum TCII over time for intervention group participants.

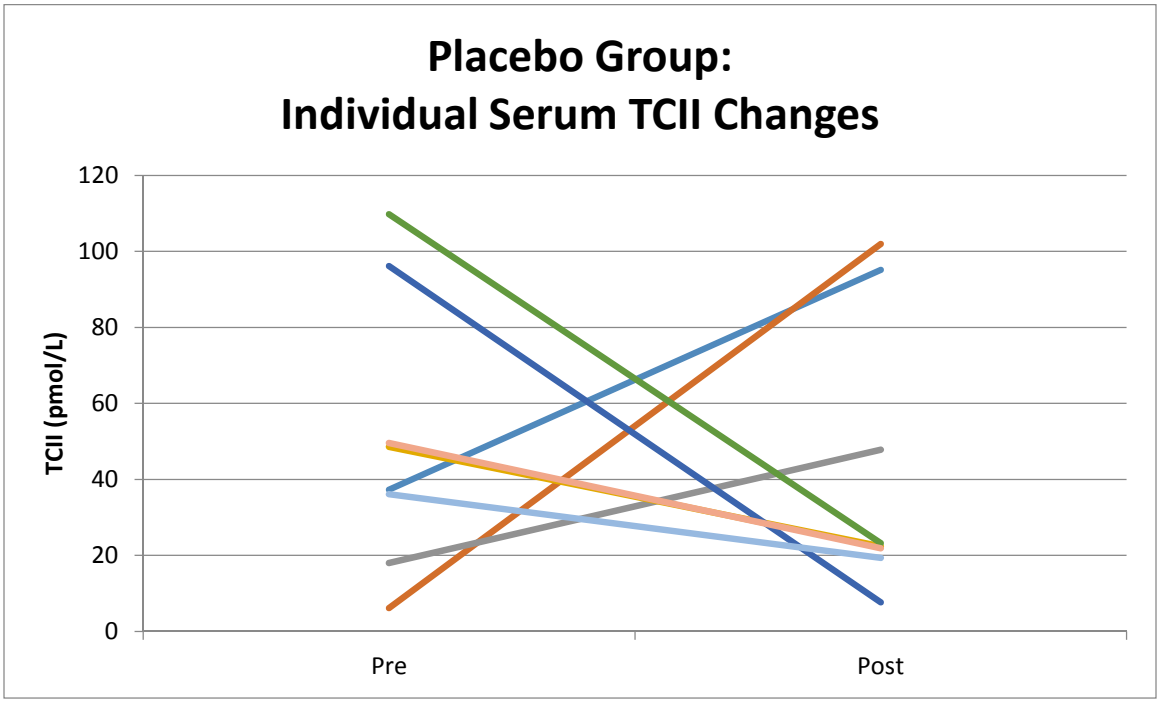

Figure 14: Changes in serum TCII over time for placebo group participants.

When the time*group interactions were analyzed for participants with a baseline serum B12 less than 303 pmol/L, the effect size for left hand adjusted increased to 0.281, however significance was not achieved (p=0.142). In addition, when controlling for depletion at baseline (less than 303 pmol/L), there was a significant time*group interaction for the Vx max outcome variable (p=0.049, $p\eta^2$ =0.250). When only the subjects with serum B12 less than 303 pmol/L were analyzed, the time*group interaction for Vx max had an effect size of 0.242, but was not significant (p=0.179). For this analysis, the intervention group consisted of 5 subjects and the placebo group consisted of 4 subjects.

CHAPTER 5

DISCUSSION

This is the first study to compare the relationship between the vitamin B12 status of vegetarians with manual dexterity, balance, and sensory perception measures. Currently, plasma vitamin B12 assessment cutoffs (148 pmol/L for total vitamin B12 concentration and 35 pmol/L for TCII concentrations) are based on non-specific anemia parameters: hemoglobin value, normal MCV, and normal reticulocyte response [15]. Further, the basis for the vitamin B12 DRI does not include data regarding neurological symptoms, arguably the most damaging and severe symptoms of deficiency. The focus on anemia parameters as indicators of B12 deficiency is likely due to the anemia that is typically the first clinically recognizable symptom in vitamin B12 deficiency. However, neurological complications also occur with vitamin B12 deficiency and can, if untreated, lead to frank dementia and death. These trials examined the relationship between plasma total vitamin B12 concentrations, TCII concentrations, and neurological measures.

Cross-sectional

The results of this study revealed a moderate correlation ($R=-0.351$, $p=0.031$) between serum B12 and left-hand manual dexterity scores on the functional dexterity test. No other correlations were noted between other outcome variables and serum B12 or TCII. Furthermore, when comparing participants who reported regularly supplementing with vitamin B12 and those who did not report this, the supplementing group had significantly better serum B12 ($p=0.017$), TCII ($p=0.041$), and left-hand manual dexterity than the non-supplementing group ($p=0.005$). Although the correlation between B12 and

left-hand manual dexterity was strengthened after controlling for hand dominance, there were only 2 two left-hand dominant subjects. Thus, these results only have external validity in a right-hand dominant population. Nonetheless, the relationship between vitamin B12 and left-hand manual dexterity appears to be consistent in this portion of the study.

Although there were few significant correlations between biochemical results and outcome variables, there were several group differences of note in outcome variables. Most notably, the length of diet adherence appears to have an effect on dexterity but not vibration sensitivity. Differences in neurological outcomes were compared between the short-term (3-5 years) and long-term (>5 years) diet adherence groups due to the liver's ability to store vitamin B12 and sustain the body in times of dietary depletion up to 3-5 years [20]. This is evidenced by a significant difference in four of the five Purdue Pegboard variables between the short-term vegetarians and vegans (3-5 years of diet adherence) and long-term vegetarians and vegans (>5 years of diet adherence). In addition, there was also a difference between the long-term and short-term groups for the Functional Dexterity Test left hand adjusted score ($p=0.042$). When comparing the short-term and the long-term diet adherence groups, the mean test completion time for the short-term diet group was nearly six seconds shorter than the long-term diet adherence group ($p=0.042$). According to Sartorio et al., the 50th percentile reference norm for adjusted non-dominant (left in this study sample) hand is 23.2 seconds for males and 23.9 seconds for females between 20 and 49 years of age [91]. The average score for the high B12 group was 23.4 seconds, which is very similar to the 50th percentile reference norm for this age

range. However, the average score for the low B12 group was 29.7 seconds, closer to the 84th percentile reference norm (29.8 seconds for males, 32.6 seconds for females). A difference of six seconds in a 24-second task is noteworthy, as this may indicate declines in skills such as handwriting and typing [55]. The short-term diet adherence group also had significantly better scores on the Purdue Pegboard left-hand dexterity test (p=0.003). Although the difference between the two groups (1.5 pins) does not appear as striking as that of the left hand Functional Dexterity Test, subtle decreases in fine dexterity may affect daily activities such as buttoning a shirt or texting. Although the short-term diet adherence group had a higher mean serum B12 (390 pmol/L) than the long-term diet adherence group (331 pmol/L), there were no significant differences in serum B12 or TCII between the two diet length groups. This suggests that these functional differences in fine dexterity are due to a different underlying factor not measured in this study. For example, it is possible that a metabolite of the vitamin B12 metabolic cycle, such as homocysteine, is a mediator for the impact of vitamin B12 on nervous system function. One study found that children whose mothers had high maternal homocysteine concentrations at 26 weeks gestation had significantly lower (worse) dexterity scores than children whose mothers had normal plasma homocysteine at 26 weeks gestation [92]. Although plasma homocysteine tends to be elevated in vegetarians and vegans [93], plasma homocysteine concentration is impacted by other nutrients such as folate and vitamin B6 [20].

While the Purdue Pegboard likely requires proper functioning of sensory nerves and motor nerves, the vibration sensitivity testing likely requires only proper functioning

of sensory nerves (as there is no movement involved in sensing the vibration intensity). Thus the group difference in the dexterity variables, but not the vibration sensitivity, suggests possible motor nerve differences between the diet adherence groups. Due to the cost and need for a physician, however, nerve conduction velocities were not measured in this study. Thus, the difference between a motor and sensory nerves is merely speculative.

As nutrient interactions can often affect the bioavailability of a vitamin or mineral, it is important to examine the nutrient composition of supplements. Yamada et al. found that compounds such as sugar, iron, and vitamin C promote the conversion of vitamin B12 to biologically inactive B12 analogues. In addition, liquid supplements are also sensitive to storage time, light, and temperature, however solid supplements in this study were not as sensitive to environmental conditions as their liquid counterparts [94]. However, due to the possibility of vitamin B12 degradation, group differences were examined between participants who obtained supplemental vitamin B12 from multivitamins and those who obtained vitamin B12 from B-complex or 'stand-alone' B12 supplements. Although there were several significant group differences, they were not consistent. For example, the group who reported taking combined supplements had better scores for some balance markers (average velocity, path length, and area effective) and left-hand manual dexterity, but worse left-hand fine dexterity. In addition, there was no significant difference between vitamin B12 and TCII between the two groups, although serum B12 for the combined supplement group (412 pmol/L) was lower than that of the B12/B12 complex group (446 pmol/L). The validity of the statistical analysis

for this group comparison is diminished by low sample sizes for the combined supplement group (n=10) and the B12/B12 complex group (n=11). These results suggest the possibility that a secondary nutrient (possibly found in combined supplements) may be the cause for the group difference in balance and dexterity markers, as serum B12 did not vary significant across groups. However, more research with larger sample sizes examining the functional consequences of vitamin B12 supplemental source is needed to confirm this speculation.

Group differences in outcome scores using a new cut-off point for defining deficiency based on the 50% percentile of serum B12 (303 pmol/L) for the 38 participants were also examined. In participants grouped by the 50th percentile for serum vitamin B12 concentrations (303 pmol/L), the high serum B12 group had significantly better left-hand manual dexterity scores (p=0.034) than the low serum B12 group (which contained no deficient individuals). Thus left-hand dexterity appears to be directly related to vitamin B12 concentrations, as well as to the length of vegetarian or vegan diet adherence. This research supports recent research that concludes that true vitamin B12 deficiency cannot be ruled out in cases of serum concentrations between 156 and 450 pmol/L [53]. This analysis also revealed a significant difference for area95 between the high and low serum B12 groups (p=0.030; data square root transformed to achieve normality). This, combined with the moderate effect size of 0.170 for Vx max in the intervention portion of the study, is evidence for the need of further research on the effects of vitamin B12 nutriture on balance measures, perhaps in the elderly or deficient individuals. However, one recent study which examined neurological outcomes after

supplementation in older adults also found no changes in functional neurological outcomes upon supplementation of vitamin B12 [95]. As none of the subjects were deficient according to the current definition of vitamin B12 deficiency (<148 pmol/L), the negative consequences of low nutriture above deficiency provided also further evidence for the need to reexamine the vitamin B12 deficiency cut-offs.

The strengthened relationship between vitamin B12 depletion and balance at lower serum B12 nutriture suggests that balance is the first neurological outcome affected by inadequate vitamin B12. Similar results were observed in a recent study where investigators did not observe a pronounced change in the nerve conduction speed in the median nerve in the arm, but did document a change in the nerve conduction speed in the sural nerve on the leg. Brito et al. suggested that this was possibly due to vitamin B12 deficiency demyelination affecting larger afferent peripheral nerves [79], rather than the smaller branches located in the fingertips.

Intervention

The results of the intervention portion of this study revealed no significant change in outcome variables over time between the intervention and placebo groups. Although there were several large and moderate effect sizes, the change in these variables were not significant. However, when controlling for B12 depletion (<303 pmol/L) at baseline, the intervention group's Vx max scores improved significantly more than the placebo groups scores (p=0.049). In addition, when examining the changes in neurological outcomes over time between the two groups, there was a large effect size ($p\eta^2$ =0.242) related to the change in Vx max favoring the intervention group (p=0.179). When analyzing this group

difference using Cohen's D, the effect size is moderate (Cohen's D = 0.553). As the sample size was small for this analysis (n=9), it is again important to examine the effect size in addition to the p-value (p=0.113). When examining only those participants whose serum B12 was less than 303 pmol/L at baseline, the effect size regarding the difference between the intervention and placebo groups over time was strengthened ($p\eta^2$ =0.281). This again suggests that improvements in neurological function, particularly balance, are more profound for individuals who are in a state of depletion rather than B12 excess.

Change in nervous system markers of the lower limbs, but not the upper limbs, is consistent with pervious literature. Similar results were observed in a recent study where investigators did not observe a pronounced change in the nerve conduction speed in the median nerve in the arm, but did document a change in the nerve conduction speed in the sural nerve on the leg [79]. In addition, in cases of diabetic neuropathy, the feet are typically affected before neuropathy develops in upper extremeties [96].

In addition to the lack of time*group interactions related to outcome variables, there is only one time*group interaction for biochemical markers which is lost after removing outliers from analysis. While the intervention group's serum B12 increased significantly more than the placebo group's serum B12 concentrations over time (p=0.042, $p\eta^2$=0.234, Δ=154.8 pmol/L), this significance disappeared when the outlier was removed (p=0.061, $p\eta^2$=0.214, Δ=109.8 pmol/L). With the outlier removed, however, the change in serum B12 (109.8 pmol/L) is trending towards significance. When removing the serum B12 outlier participant from TCII analysis, the change in serum TCII over time between groups remained not significant (p=0.213, $p\eta^2$=0.101).

However, the TCII data point from the participant who had a very high serum B12 is not an outlier and should remain in the analysis.

The non-significant increase in serum B12 (with the outlier removed) and TCII after 8 weeks of 500mcg daily supplementation (208% of the RDA) of vitamin B12 is a surprising result. However, when analyzing only those participants whose baseline serum B12 was below the 50th percentile (303 pmol/L), there was a strong effect size of 0.387 ($p=0.074$) for daily supplementation. However, in cases of small sample size such as this (5 in the intervention group and 4 in the placebo group), it is important to take both the significance and the effect size into consideration. The large, although not significant, change in serum B12 in the intervention group ($p\eta^2=0.234$) combined with the large increase in the effect size of the time*group interactions for participants whose baseline serum B12 was below the 50th percentile, suggests that vitamin B12 supplementation may have a more pronounced effect on those individuals who are in a state of vitamin B12 depletion (<303 pmol/L serum concentration). However, when using the Cohen's D calculation, there is only a small effect size (Cohen's D = 0.465) when supplementing vitamin B12 in the vitamin B12 depletion group.

This lack of a significant increase in serum B12 (after outlier was removed) and TCII over time also suggests that 8 weeks of oral supplementation is not a long enough period to create significant change in plasma vitamin B12. Eight weeks was selected for the trial length due to the typical resolution of hematological abnormalities within this time frame [8]. However, there is a lack of consistent information in the literature regarding the length of time for resolution of neurological symptoms. In one study

examining the treatment times necessary to resolve vitamin B12 deficiency symptoms in more severe cases, investigators found that only 54% of patients responded to 13 months of treatment with vitamin B12 injections [97]. However, no studies have examined the supplementation to reverse early neurological symptoms. As these subtle changes are difficult to recognize, healthcare providers typically see patients who are later in symptom progression. For example, one study found that patients had been experiencing symptoms of vitamin B12 deficiency for an average of 10 months before being seen by a healthcare professional [97]. Thus, it is likely that improvements in neurological measures would require long-term supplementation, possibly greater than 13 months.

Conclusion

We conclude that vitamin B12 appears to have more consistent relationships with neurological outcome variables than holo-transcobalamin II. In addition, there is a relationship between serum B12 and left-hand dexterity (in a population of right-hand dominant individuals), as well as a relationship between serum vitamin B12 and x-axis velocity in subjects whose serum vitamin B12 concentration is less than 303 pmol/L. More research is necessary to examine the relationship between vitamin B12 serum concentration and vitamin B12 supplementation on neurological outcomes, particularly balance and left-hand dexterity, in a large sample of subjects with baseline serum concentrations less than 303 pmol/L. Although this study supports the notion that there are slight functional declines in balance in states of B12 depletion, the sample size for this study is small. The functional consequences of vitamin B12 depletion (defined here as less than 303 pmol/L) need to be examined in a large-scale study to further elucidate

the clinical relevance of vitamin B12 depletion. Finally, further research needs to be conducted as to the efficacy of oral supplementation in individuals who have serum B12 concentrations in the low range of adequacy.

REFERENCES

1. Pawlak R, Parrott SJ, Raj S, Cullum-Dugan D, Lucus D. How prevalent is vitamin B12 deficiency among vegetarians? Nutr Rev. 2013;71(2):110-117.

2. Karabudak E, Kiziltan G, Cigerim N. A comparison of some of the cardiovascular risk factors in vegetarian and omnivorous turkish females. Journal of Human Nutrition and Dietetics. 2008;21(1):13-22.

3. Krajcovicova-Kudlackova M, Blazicek P, Mislanova C, Valachovicova M, Paukova V, Spustova V. Nutritional determinants of plasma homocysteine. Bratisl Lek Listy. 2007;108(12):510-515.

4. Herrmann W, Schorr H, Purschwitz K, Rassoul F, Richter V. Total homocysteine, vitamin B12, and total antioxidant status in vegetarians. Clin Chem. 2001;47(6):1094.

5. Evatt ML, Mersereau PW, Bobo JK, Kimmons J, Williams J,. Table 3: Prevalence of vitamin B12 serum levels for the U.S. population by age, national health and nutrition examination survey 2001-2004.. Centers for Disease Control and Prevention Web site. http://www.cdc.gov/ncbddd/b12/table3.html. Updated 2009. Accessed 3/9, 2015.

6. Herbert V. Staging vitamin B-12 (cobalamin) status in vegetarians. Am J Clin Nutr. 1994;59(5 Suppl):1213S.

7. Kumar N. Neurologic aspects of cobalamin (B12) deficiency. Handb Clin Neurol. 2014;120:915-926.

8. Briani C, Dalla Torre C, Citton V, et al. Cobalamin deficiency: Clinical picture and radiological findings. Nutrients. 2013;5(11):4521-4539.

9. Hunt A, Harrington D, Robinson S. Vitamin B12 deficiency. BMJ. 2014;349:g5226.

10. Bachman P, Niendam TA, Jalbrzikowkski M, et al. Processing speed and neurodevelopment in adolescent-onset psychosis: Cognitive slowing predicts social function. J Abnorm Child Psychol. 2012;40(4):645-654.

11. Butt AM, Fern RF, Matute C. Neurotransmitter signaling in white matter. Glia. 2014;62(11):1762-1779.

12. Koppelmans V, Hirsiger S, Merillat S, Jancke L, Seidler RD. Cerebellar gray and white matter volume and their relation with age and manual motor performance in healthy older adults. Hum Brain Mapp. 2015.

13. Yang C, Hsu H, Lu C, Chao Y, Chiu H, Kuo L. The associations among hand dexterity, functional performance, and quality of life in diabetic patients with

neuropathic hand from objective- and patient-perceived measurements. Quality of Life Research. 2015;24(1):213-221.

14. Herrmann W, Obeid R. Causes and early diagnosis of vitamin B12 deficiency. Deutsches Arzteblatt international. 2008;105(40):680-685.

15. Subcommittee on Upper Reference Levels of Nutrients, Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, Institute of Medicine (U.S.), Panel on Folate, Other B Vitamins,and Choline. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. Washington, D.C: National Academy Press; 1998.

16. BROQUIST HP. Water-soluble vitamins. I. folic acid, B12 group, choline. Annu Rev Biochem. 1958;27(3):285.

17. Herbert V. Vitamin B-12: Plant sources, requirements, and assay. Am J Clin Nutr. 1988;48(3 Suppl):852.

18. TRUSWELL AS. Vitamin B12. Nutrition & Dietetics. 2007;64(s4 The Role of):S120-S125.

19. Dietary supplement fact sheet: Vitamin B12. Office of Dietary Supplements, National Institutes of Health Web site. http://ods.od.nih.gov/factsheets/VitaminB12-QuickFacts/#h8. Updated 2011. Accessed 09/26, 2013.

20. Rush EC, Katre P, Yajnik CS. Vitamin B12: One carbon metabolism, fetal growth and programming for chronic disease. Eur J Clin Nutr. 2014;68(1):2-7.

21. Quadros EV. Advances in the understanding of cobalamin assimilation and metabolism. Br J Haematol. 2010;148(2):195-204.

22. Shipton MJ, Thachil J. Vitamin B12 deficiency - A 21st century perspective. Clin Med (Lond). 2015;15(2):145-150.

23. Seetharam B. Receptor-mediated endocytosis of cobalamin (vitamin B12). Annu Rev Nutr. 1999;19(1):173-195. doi: 10.1146/annurev.nutr.19.1.173.

24. Loikas S, Lopponen M, Suominen P, et al. RIA for serum holo-transcobalamin: Method evaluation in the clinical laboratory and reference interval. Clin Chem. 2003;49(3):455-462. doi: 10.1373/49.3.455.

25. Rothenberg SP, Quadros EV. Transcobalamin II and the membrane receptor for the transcobalamin II-cobalamin complex. Baillieres Clin Haematol. 1995;8(3):499-514. doi: 10.1016/S0950-3536(05)80218-5.

26. Refsum H, Johnston C, Guttormsen AB, Nexo E. Holotranscobalamin and total transcobalamin in human plasma: Determination, determinants, and reference values in healthy adults. Clin Chem. 2006;52(1):129-137. doi: 10.1373/clinchem.2005.054619.

27. Bhat DS, Thuse NV, Lubree HG, et al. Increases in plasma holotranscobalamin can be used to assess vitamin B-12 absorption in individuals with low plasma vitamin B-12. J Nutr. 2009;139(11):2119-2123. doi: 10.3945/jn.109.107359.

28. Pawlak R. Is vitamin B12 deficiency a risk factor for cardiovascular disease in vegetarians? Am J Prev Med. 2015;48(6):e11-e26. doi: 10.1016/j.amepre.2015.02.009.

29. Key TJ, Appleby PN, Davey GK, Allen NE, Spencer EA, Travis RC. Mortality in british vegetarians: Review and preliminary results from EPIC-oxford. Am J Clin Nutr. 2003;78(3 Suppl):533S.

30. Orlich MJ, Singh PN, Sabate J, et al. Vegetarian dietary patterns and mortality in adventist health study 2. JAMA Internal Medicine. 2013;173(13):1230-1238. doi: 10.1001/jamainternmed.2013.6473.

31. Messika AH, Kaluski DN, Lev E, et al. Nutrigenetic impact of daily folate intake on plasma homocysteine and folate levels in patients with different methylenetetrahydrofolate reductase genotypes. European Journal of Cardiovascular Prevention & Rehabilitation. 2010;17(6):701-705. doi: 10.1097/hjr.0b013e32833a1cb5.

32. Refsum H, Nurk E, Smith AD, et al. The hordaland homocysteine study: A community-based study of homocysteine, its determinants, and associations with disease. J Nutr. 2006;136(6):1731S-1740S.

33. Whincup PH, Refsum H, Perry IJ, et al. Serum total homocysteine and coronary heart disease: Prospective study in middle aged men. Heart. 1999;82(4):448-454. doi: 10.1136/hrt.82.4.448.

34. Selhub J, Morris MS, Jacques PF. In vitamin B?? deficiency, higher serum folate is associated with increased total homocysteine and methylmalonic acid concentrations. Proc Natl Acad Sci U S A. 2007;104(50):19995-20000. doi: 10.1073/pnas.0709487104.

35. Jacques P, Wilson PWF, Selhub J, Tucker KL, Mahnken B. Folic acid fortification of the food supply: Potential benefits and risks for the elderly population. JAMA. 1996;276(23):1879-1885. doi: 10.1001/jama.1996.03540230029031.

36. Morris M, Jacques P, Rosenberg I, Selhub J. Folate and vitamin B-12 status in relation to anemia, macrocytosis, and cognitive impairment in older americans in the age of folic acid fortification. Am J Clin Nutr. 2007;85(1):193-200.

37. Fraser WD, Milan AM. Vitamin D assays: Past and present debates, difficulties, and developments. Calcif Tissue Int. 2013;92(2):118-127. doi: 10.1007/s00223-012-9693-3.

38. Vedral JL, Institute of Medicine Staff, ebrary I. Dietary reference intakes: For calcium, phosphorus, magnesium, vitamin D, and fluoride. Washington, D.C: National Academy Press; 1997; 1999.

39. Supertracker. United States Department of Agriculture Web site. https://www.supertracker.usda.gov/default.aspx. Accessed 3/14, 2016.

40. Pawlak R, Parrott SJ, Raj S, Cullum-Dugan D, Lucus D. How prevalent is vitamin B12 deficiency among vegetarians? Nutr Rev. 2013;71(2):110-117.

41. Herrmann W, Schorr H, Obeid R, Geisel J. Vitamin B-12 status, particularly holotranscobalamin II and methylmalonic acid concentrations, and hyperhomocysteinemia in vegetarians. Am J Clin Nutr. 2003;78(1):131.

42. Elmadfa I, Singer I. Vitamin B-12 and homocysteine status among vegetarians: A global perspective. Am J Clin Nutr. 2009;89(5):1693S.

43. Gilsing AMJ, Crowe FL, Lloyd-Wright Z, et al. Serum concentrations of vitamin B12 and folate in british male omnivores, vegetarians and vegans: Results from a cross-sectional analysis of the EPIC-oxford cohort study. Eur J Clin Nutr. 2010;64(9):933-939. doi: 10.1038/ejcn.2010.142.

44. Herrmann W, Schorr H, Obeid R, Geisel J. Vitamin B-12 status, particularly holotranscobalamin II and methylmalonic acid concentrations, and hyperhomocysteinemia in vegetarians. Am J Clin Nutr. 2003;78(1):131.

45. Honzik T, Adamovicova M, Smolka V, Magner M, Hruba E, Zeman J. Clinical presentation and metabolic consequences in 40 breastfed infants with nutritional vitamin B12 deficiency--what have we learned? European journal of paediatric neurology : EJPN : official journal of the European Paediatric Neurology Society. 2010;14(6):488-495. doi: 10.1016/j.ejpn.2009.12.003.

46. Goraya JS, Kaur S, Mehra B. Neurology of nutritional vitamin B12 deficiency in infants: Case series from india and literature review. J Child Neurol. 2015;30(13):1831.

47. Tucker KL. Vegetarian diets and bone status. Am J Clin Nutr. 2014;100 Suppl 1(1):329S. doi: 10.3945/ajcn.113.071621.

48. Kok DEG, Dhonukshe-Rutten RAM, Lute C, et al. The effects of long-term daily folic acid and vitamin B12 supplementation on genome-wide DNA methylation in elderly subjects. Clinical epigenetics. 2015;7(1):121. doi: 10.1186/s13148-015-0154-5.

49. Bell KN, Oakley J,Godfrey P. Tracking the prevention of folic acid-preventable spina bifida and anencephaly. Birth defects research.Part A, Clinical and molecular teratology. 2006;76(9):654-657. doi: 10.1002/bdra.20304.

50. Ray JG, Wyatt PR, Thompson MD, et al. Vitamin B?? and the risk of neural tube defects in a folic-acid-fortified population. Epidemiology. 2007;18(3):362-366. doi: 10.1097/01.ede.0000257063.77411.e9.

51. Wang Z, Shang X, Zhao Z. Low maternal vitamin B12 is a risk factor for neural tube defects: A meta-analysis. Journal of Maternal-Fetal and Neonatal Medicine. 2012;25(4):389-394. doi: 10.3109/14767058.2011.580800.

52. Stabler SP. Clinical practice. vitamin B12 deficiency. N Engl J Med. 2013;368(2):149.

53. Obeid R, Herrmann W. Holotranscobalamin in laboratory diagnosis of cobalamin deficiency compared to total cobalamin and methylmalonic acid. Clinical chemistry and laboratory medicine : CCLM / FESCC. 2007;45(12):1746-1750.

54. Yancosek KE, Howell D. A narrative review of dexterity assessments. Journal of Hand Therapy. 2009;22(3):258-270. doi: 10.1016/j.jht.2008.11.004.

55. Sartorio F, Bravini E, Vercelli S, et al. The functional dexterity test: Test-retest reliability analysis and up-to date reference norms. Journal of hand therapy : official journal of the American Society of Hand Therapists. 2013;26(1):62. doi: 10.1016/j.jht.2012.08.001.

56. Tiffin J, Asher EJ. The purdue pegboard: Norms and studies of reliability and validity. J Appl Psychol. 1948;32(3):234-247.

57. Buddenberg LA, Davis C. Test-retest reliability of the purdue pegboard test. Am J Occup Ther. 2000;54(5):555-558. doi: 10.5014/ajot.54.5.555.

58. Chaudhry H, Bukiet B, Ji Z, Findley T. Measurement of balance in computer posturography: Comparison of methods-A brief review. Journal of Bodywork & Movement Therapies. 2011;15(1):82-91.

59. Clark RA, Bryant AL, Pua Y, McCrory P, Bennell K, Hunt M. Validity and reliability of the nintendo wii balance board for assessment of standing balance. Gait Posture. 2010;31(3):307-310.

60. Eftekhar-Sadat B, Azizi R, Aliasgharzadeh A, Toopchizadeh V, Ghojazadeh M. Effect of balance training with biodex stability system on balance in diabetic neuropathy. Therapeutic advances in endocrinology and metabolism. 2015;6(5):233-240. doi: 10.1177/2042018815595566.

61. Kneis S, Wehrle A, Freyler K, et al. Balance impairments and neuromuscular changes in breast cancer patients with chemotherapy-induced peripheral neuropathy. Clinical Neurophysiology. 2015. doi: 10.1016/j.clinph.2015.07.022.

62. Fortaleza ACdS, Chagas EF, Ferreira DMA, et al. Postural control and functional balance in individuals with diabetic peripheral neuropathy. Brazilian Journal of Kinanthropometry and Human Performance. 2013;15(3):305-314. doi: 10.5007/1980-0037.2013v15n3p305.

63. Swart KMA, Ham AC, van Wijngaarden JP, et al. A randomized controlled trial to examine the effect of 2-year vitamin B12 and folic acid supplementation on physical performance, strength, and falling: Additional findings from the B-PROOF study. Calcif Tissue Int. 2016;98(1):18-27. doi: 10.1007/s00223-015-0059-5.

64. Simpson JL, Bailey LB, Pietrzik K, Shane B, Holzgreve W. Micronutrients and women of reproductive potential: Required dietary intake and consequences of dietary deficiency or excess. part I - folate, vitamin B12, vitamin B6. Journal of Maternal-Fetal and Neonatal Medicine. 2010;23(12):1323-1343. Error! Hyperlink reference not valid.. doi: 10.3109/14767051003678234.

65. Mahajan SK, Aundhakar SC. A study of the prevalence of serum vitamin B12 and folic acid deficiency in western maharashtra. Journal of family medicine and primary care. 2015;4(1):64. doi: 10.4103/2249-4863.152255.

66. Ssonko M, Ddungu H, Musisi S. Low serum vitamin B12 levels among psychiatric patients admitted in butabika mental hospital in uganda. BMC research notes. 2014;7(1):90-90. doi: 10.1186/1756-0500-7-90.

67. Roos D. The vibration verception threshold in gastrectomized patients with low serum B12 . Acta Neurol Scandinav. 1977;56:551-562.

68. Talaei A, Siavash M, Majidi H, Chehrei A. Vitamin B12 may be more effective than nortriptyline in improving painful diabetic neuropathy. Int J Food Sci Nutr. 2009;60(s5):71-76. doi: 10.1080/09637480802406153.

69. Kibirige D, Mwebaze R. Vitamin B12 deficiency among patients with diabetes mellitus: Is routine screening and supplementation justified? Journal of Diabetes and Metabolic Disorders. 2013;12(1):17-17. doi: 10.1186/2251-6581-12-17.

70. Gerr FE, Letz R. Reliability of a widely used test of peripheral cutaneous vibration sensitivity and a comparison of two testing protocols. Br J Ind Med. 1988;45(9):635-639.

71. Pambianco G, Costacou T, Strotmeyer E, Orchard TJ. The assessment of clinical distal symmetric polyneuropathy in type 1 diabetes: A comparison of methodologies from the pittsburgh epidemiology of diabetes complications cohort. Diabetes Res Clin Pract. 2011;92(2):280-287. doi: 10.1016/j.diabres.2011.02.005.

72. Mete T, Aydin Y, Saka M, et al. Comparison of efficiencies of michigan neuropathy screening instrument, neurothesiometer, and electromyography for diagnosis of diabetic neuropathy. International Journal of Endocrinology. 2013;2013:1-7. doi: 10.1155/2013/821745.

73. Pambianco G, Costacou T, Strotmeyer E, Orchard TJ. The assessment of clinical distal symmetric polyneuropathy in type 1 diabetes: A comparison of methodologies from the pittsburgh epidemiology of diabetes complications cohort. Diabetes Res Clin Pract. 2011;92(2):280-287. doi: 10.1016/j.diabres.2011.02.005.

74. Resnick HE, Vinik AI, Heimovitz HK, Brancati FL, Guralnik JM. Age 85+ years accelerates large-fiber peripheral nerve dysfunction and diabetes contributes even in the oldest-old: The women's health and aging study. The journals of gerontology.Series A, Biological sciences and medical sciences. 2001;56(1):M25-M31. doi: 10.1093/gerona/56.1.M25.

75. Szczyrba S, Kozera GM, Neubauer-Geryk J, Wolnik B, Nyka WM, Bieniaszewski L. Diabetic symmetric polyneuropathy is associated with increased aortal stiffening but not cerebral angiopathy in type 1 diabetes. J Diabetes Complications. 2015;29(1):73-76. doi: 10.1016/j.jdiacomp.2014.10.002.

76. Forsyth Pa, Balmaceda C, Peterson K, Seidman AD, Brasher P, Deangelis LM. Prospective study of paclitaxel-induced peripheral neuropathy with quantitative sensory testing. J Neurooncol. 1997;35(1):47-53. doi: 10.1023/A:1005805907311.

77. Oh J, Zackowski K, Chen M, et al. Multiparametric MRI correlates of sensorimotor function in the spinal cord in multiple sclerosis. Multiple Sclerosis Journal. 2013;19(4):427-435. doi: 10.1177/1352458512456614.

78. Zackowski KM, Smith SA, Reich DS, et al. Sensorimotor dysfunction in multiple sclerosis and column-specific magnetization transfer-imaging abnormalities in the spinal cord. Brain. 2009;132(5):1200-1209. doi: 10.1093/brain/awp032.

79. Brito A, Verdugo R, Hertrampf E, et al. Vitamin B-12 treatment of asymptomatic, deficient, elderly chileans improves conductivity in myelinated peripheral nerves, but high serum folate impairs vitamin B-12 status response assessed by the combined indicator of vitamin B-12 status. Am J Clin Nutr. 2016;103(1):250-257.

80. Hvas A, Nexo E. Diagnosis and treatment of vitamin B12 deficiency--an update. Haematologica. 2006;91(11):1506.

81. Malpas JS, Spray GH, Witts LJ. Serum folic-acid and vitamin-B12 levels in anticonvulsant therapy. The British Medical Journal. 1966;1(5493):955-957.

82. Baer J, Peter MS. Vitamin B12 assessment and intervention in younger adult women. The Journal for Nurse Practitioners. 2011;7(2):117-122.

83. Morris N, Lynch K, Greenberg SA. Severe motor neuropathy or neuronopathy due to nitrous oxide toxicity after correction of vitamin B12 deficiency. Muscle Nerve. 2015;51(4):614-616.

84. Dietary reference intakes (DRIs): Recommended intakes for individuals, total water and macronutrients. http://www.nap.edu/openbook.php?record_id=10490&page=1324. Accessed 10/15, 2012.

85. Bonaa KH, Rasmussen K, Njolstad I, et al. Homocysteine lowering and cardiovascular events after acute myocardial infarction. N Engl J Med. 2006;354(15):1578-1588.

86. Lonn E, Held C, Genest J, Jacques, et al. Homocysteine lowering with folic acid and B vitamins in vascular disease. N Engl J Med. 2006;354(15):1567-1577.

87. Das K, Manusselis C, Herbert V. Determination of vitamin B12 (cobalamin) in serum and erythrocytes by radioassay, and of holo-transcobalamin II (holo-TCII) and holo-hepatocorrin (holo-TCI and III) in serum by adsorbing holo-TCII on microfine silica. J Nutr Biochem. 1991;2:455-463.

88. Telles S, Yadav A, Kumar N, Sharma S, Visweshwaraiah NK, Balkrishna A. Blood pressure and purdue pegboard scores in individuals with hypertension after alternate nostril breathing, breath awareness, and no intervention. Medical science monitor : international medical journal of experimental and clinical research. 2013;19:61.

89. Martin CL, Waberski BH, Pop-Busui R, et al. Vibration perception threshold as a measure of distal symmetrical peripheral neuropathy in type 1 diabetes: Results from the DCCT/EDIC study. Diabetes Care. 2010;33(12):2635-2641. doi: 10.2337/dc10-0616.

90. Sahin S, Karsidag S, Ayalp S, Sengul A, Us O, Karsidag K. Determination of nerve conduction abnormalities in patients with impaired glucose tolerance. Neurological Sciences. 2009;30(4):281-289.

91. Sartorio F, Bravini E, Vercelli S, et al. The functional dexterity test: Test-retest reliability analysis and up-to date reference norms. Journal of hand therapy : official journal of the American Society of Hand Therapists. 2013;26(1):62-68. doi: 10.1016/j.jht.2012.08.001.

92. Tamura T, Goldenberg RL, Chapman VR, Johnston KE, Ramey SL, Nelson KG. Folate status of mothers during pregnancy and mental and psychomotor development of their children at five years of age. Pediatrics. 2005;116(3):703-708. doi: 10.1542/peds.2004-2189.

93. Elmadfa I, Singer I. Vitamin B-12 and homocysteine status among vegetarians: A global perspective. Am J Clin Nutr. 2009;89(5):1693S.

94. Yamada K, Shimodaira M, Chida S, et al. Degradation of vitamin B12 in dietary supplements. Int J Vitam Nutr Res. 2008;78(4-5):195-203.

95. Dangour AD, Allen E, Clarke R, et al. Effects of vitamin B-12 supplementation on neurologic and cognitive function in older people: A randomized controlled trial. Am J Clin Nutr. 2015;102(3):639-647. doi: 10.3945/ajcn.115.110775.

96. Coppini DV, Best C. A case of hand ulceration in the diabetic foot clinic--a reminder of hand neuropathy in 'at risk' patients. Diabetic medicine : a journal of the British Diabetic Association. 2000;17(9):682.

97. Aaron S, Kumar Sudhir, Vijayan J, Jacob J, Alexander M,Gnanamuthu C. Clinical and laboratory features and response to treatment in patients presenting with vitamin B12 deficiency-related neurological syndromes. Neurol India. 2005;53(1):55-58.

APPENDIX A

IRB APPROVAL FORM

APPROVAL: EXPEDITED REVIEW

Carol Johnston
SNHP: Nutrition
602/827-2265
CAROL.JOHNSTON@asu.edu

Dear Carol Johnston:

On 9/24/2015 the ASU IRB reviewed the following protocol:

Type of Review:	Initial Study
Title:	Vitamin B12 and health biomarkers in young adult vegetarians
Investigator:	Carol Johnston
IRB ID:	STUDY00003188
Category of review:	(2)(a) Blood samples from healthy, non-pregnant adults, (7)(b) Social science methods, (2)(b) Blood samples from others, (7)(a) Behavioral research
Funding:	Name: Graduate College
Grant Title:	
Grant ID:	
Documents Reviewed:	• flyer, Category: Recruitment Materials; • consent (trial 1), Category: Consent Form; • Flyer (print), Category: Recruitment Materials; • Health history questionnaire, Category: Screening forms; • protocol, Category: IRB Protocol; • consent (trial 2), Category: Consent Form; • diet recall, Category: Measures (Survey questions/Interview questions /interview guides/focus group questions);

The IRB approved the protocol from 9/24/2015 to 9/23/2016 inclusive. Three weeks before 9/23/2016 you are to submit a completed Continuing Review application and required attachments to request continuing approval or closure.

If continuing review approval is not granted before the expiration date of 9/23/2016 approval of this protocol expires on that date. When consent is appropriate, you must use final, watermarked versions available under the “Documents” tab in ERA-IRB.

In conducting this protocol you are required to follow the requirements listed in the INVESTIGATOR MANUAL (HRP-103).

Sincerely,

IRB Administrator

cc:
Taylor Arnold

APPENDIX B

FORCE PLATE INSTRUCTIONS

1. Force Plate Set Up
    a. Make sure 30° tape is on force plate
    b. Set up force plate and laptop and connect to power source using the extension cord and power strip
    c. Ensure the force plate is connected to COM4 on the laptop
    d. Open Balance Clinic
    e. Ensure COM4 is selected in the "setup" section of BC
    f. Calibrate the force plate using the "digital XXXX .cal" file
    g. Select the B12CS protocol
2. Posturography Analysis
    a. Click "select subject" and "add new" to enter each subject's information
    b. Enter their subject number as the first name and the date (DD.MM.YY) as the last name
    c. Enter the subject's height and weight as measured at the anthropometrics station
    d. Click "OK"
    e. Make sure that subject is selected, click "OK"
    f. Zero the platform
    g. Ask the subject to remove their shoes and socks
    h. Ask the subject to stand on the platform with their feet along the outside edge of the blue tape
        i. Ask subject to stand as still as possible with their arms at their side and stare at the designated marker on the wall straight ahead of them (i.e. no talking, no moving their head)
        ii. Redo the test if they move their feet
    i. Begin 10 second timer
    j. Once 10 sec is finished, click "Acquire" on BC
        i. BC will run the test for 60 seconds and will notify you when the test is complete (click "OK")
    k. When the test is complete, ask the participant to step off of the force plate
    l. Click "Save Data"
    m. Sanitize the platform while the participant collects their belongings
    n. Export the data immediately to Google Doc

APPENDIX C

VIBRATRON II PARTICIPANT SHEET

1. Ensure that module A is connected to socket A and module B is connected to socket B. Make sure dampening pads are under each module.
2. Turn on the machine and turn the operating range to 'high resolution'.
3. Adjust the output to the highest level (7.0-7.2).
4. Allow participants to feel vibration. Provide instructions for the test while demonstrating.
    a. "Please press your finger against each rod in sequence for approximately one second. During each trial you will be allowed to touch the rods only once. Only one of the rods will be vibrating and you must decide whether it's A or B. The task will become increasingly more difficult and I understand that you will be guessing on many of the trials."
    b. When I say "OK", you will sequentially touch the top of each rod with the index finger on your dominant hand for one second each. After doing so, you will tell me which module you believe is vibrating.
    c. The ideal force to touch the rod is just enough to blanch (make yellow) your fingernail (or surrounding skin if their nail is painted).
    d. Try to be as consistent as possible with the pressure that you use to touch each module.
5. Make sure the subject cannot see the screen or switch labels.
6. Begin a trial run where you flip the real switch and the dummy switch a few times. If the participant correctly identifies the vibrating rod 100% of the time, then begin on the highest level of high resolution. If the participant incorrectly identifies the vibrating module, the switch to low resolution and begin at 10.0, then 15.0, and so on.
7. Begin the trial. Switch between A & B randomly. If you do not switch modules, flip the dummy switch.
8. After a correct identification, decrease the vibration units by 0.5.
9. If the subject makes an error, that voltage setting should be repeated 3 times.
10. If the vibrating module is correctly identified twice, then raise the units 0.5 and continue the trial.
11. If two errors are made at the same voltage consecutively, a third attempt is unnecessary.
12. All levels below 0.7 should be repeated twice.
13. Complete the test when five errors are made.

Vibration Units	Correct	Incorrect	Vibration Units	Correct	Incorrect

1. What are the vibration settings of the five errors?

2. What are the vibration settings of the five lowest correct scores?

3. Cross out the highest and lowest values of these ten scores.

4. Calculate the average of the remaining 8 scores. This is the vibration threshold (in vibration units).

5. Convert the vibration threshold from vibration units to microns using the formula below.

$$A = (0.5)X^2$$

A = peak to peak amplitude in microns
X = reading on digital display

APPENDIX D

PURDUE PEGBOARD PARTICIPANT SHEET

Purdue Pegboard Subject #:

	Trial 1	Trial 2	Trial 3	Average
Right Hand (30s)				
Left Hand (30s)				
Both Hands (30s)				
Right + Left + Both				
Assembly (60s)				

Setup

1. Separate the pins equally, half in the far left cup and half in the far right cup. Right hand gets a pin from the right. Left hand take a pin from the left.
1. RH DOMINANT: Place the washers in the cup that is right of the center of the board. Place the sleeves in the cup left of center.
2. LH DOMINANT: Place the washers in the cup that is right of the center of the board. Place the sleeves in the cup left of the center.

Pin Tests

2. DOMINANT HAND 1st: Take one pin form the far right cup, insert it into the right top hole. Keep inserting pins for 30 seconds. Don't pick up any dropped pins.
3. NON-DOMINANT HAND 2nd: Take one pin with far left cup, insert it into the top left hold. Keep inserting pins for 30 seconds. Don't pick up any dropped pins.
4. BOTH HANDS 3rd: Pins must be placed simultaneously. The pairs of pins are scored for the both hands test, not the individual pins. (Ex: 8 pins would get a score of 4, as 4 pairs will be present.)

Assembly

1. For RH dominant: Take a pin form the far right cup and insert it into the hole at the top. Grab a washer with your left and drop the washer onto the pin. Grab a collar with your right hand and place it onto the pin with the washer. Grab another washer with your left hand and drop it onto the pin to complete the assembly. Keep doing for 60 seconds.
2. For LH dominant: Repeat the same process but with the hands switched (washer in the middle-left and sleeves in the middle-right cups).
3. Scoring: Count all of the assembled parts to provide a score for the right hand. (Four points for each completed assembly. Partials count 1 point per piece.)

APPENDIX E

FUNCTIONAL DEXTERITY TEST PARTICIPANT SHEET

Functional Dexterity Test Subject #:

1. Place the board 10 cm from the edge of the table.
2. Ensure participant is comfortable.
3. Record hand dominance of participant.
4. Provide instructions for the test.
    a. Please start with your dominant hand. Start by turning the peg at the top opposite corner, turn all the pegs over as quickly as possible, turning over one row of pegs, then reversing the order in the next row, in a zigzag fashion.
    b. Do not turn your hand up to face the ceiling (supinate) or touch the board for help in turning the peg; each of these motions carries a penalty of 5 seconds. If you drop a peg, time is stopped, and a 10-second penalty is added. You then need to retrieve the peg and put it in the pegboard in the unturned position. Then continue to turn the pegs with the peg that you just put back. The clock starts where it was stopped, and the time is continued.
5. Investigator demonstrates by turning over 4 pegs.
6. Participants are allowed one practice round where they turn over all the pegs on the board once.
7. Begin test. Make sure to pause the time if the participant drops a peg. Have them insert the peg back into the board in the unturned position. Start the clock again when the participant resumes the test.

Raw Score			
Supination		x 5 seconds =	
Touching the board for help		x 5 seconds =	
Dropped peg		x 10 seconds =	
Adjusted Score			

APPENDIX F

B12 & FOLATE ASSAY PROCEDURE

Vitamin B12/Folate Assay

BRING ALL REAGENTS TO ROOM TEMP

Shake Binder vigorously!

Set-up		Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 7	Step 8	Step 9-11
Tube Name	Tube #	Add Standard/ QC or sample	Add Tracer	Vortex each tube and incubate at Room Temp x 15 min	Add Extract Reagent	Vortex each tube and incubate at Room Temp x 10 min	Add Blank Reagent	Add Binder	Vortex each tube and incubate at Room Temp x 60 min. Cover with foil place in dark.	Centrifuge everything (EXCEPT TC tube) at 4°C for 10 minutes at 3000 rpm, decant and count
Total Counts	**1,2**	-	200 µl		-		-	-		
NSB	**3,4**	200 µl STD A (0 pmol/L)	200 µl		100 µl		1000 µl	-		
Ref (BO)	**5,6**	200 µl STD A (0 pmol/L)	200 µl		100 µl			1000 µl		
Std tube B	**7,8**	200 µl of 74 pmol/L	200 µl		100 µl			1000 µl		
Std tube C	**9,10**	200 µl of 148 pmol/L	200 µl		100 µl			1000 µl		
Std tube D	**11,12**	200 µl of 296 pmol/L	200 µl		100 µl			1000 µl		
Std tube E	**13,14**	200 µl of 740 pmol/L	200 µl		100 µl			1000 µl		
Std tube F	**15,16**	200 µl of 1480 pmol/L	200 µl		100 µl			1000 µl		
Control low	**17,18**	200 µl of QC 1	200 µl		100 µl			1000 µl		
Control med	**19,20**	200 µl of QC 2	200 µl		100 µl			1000 µl		
Control high	**21,22**	200 µl of QC 3	200 µl		100 µl			1000 µl		
sample	**23,---**	200 µl of unknown	200 µl		100 µl			1000 µl		

Transcobalamin II

Mix 3 gms of Silica with 20mls of distilled water. Place on a stir plate and stir thoroughly. Check edges of beaker for silica clumping - even with a stirbar in place. Store at 4 C if not used.

While stirring, combine 100ul of slurry with 500ul of serum. Allow to sit for 10 minutes. Spin at 5000 x g for 10 minutes.
Measure the supernatant for B12 levels.

TCII is determined by subtracting the precipitated B12 from the non precipitated B12.